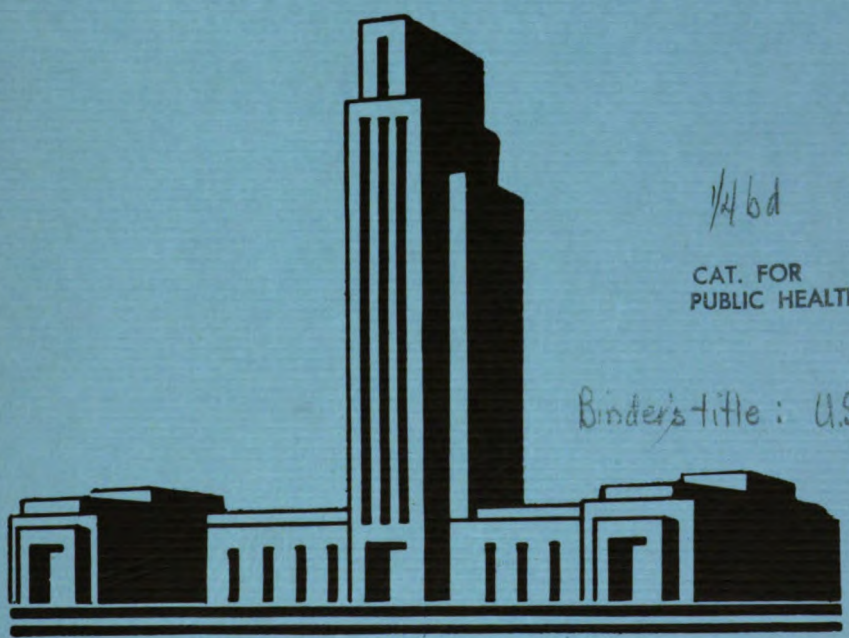
SEROLOGY



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SEROLOGY MANUAL

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SECTION I

INTRODUCTION TO SEROLOGY

Serologic tests are based on an in vitro demonstration of a reaction which takes place within the living body. Substances introduced into the living animal produce a reaction which results in altered serum proteins. The altered serum protein then reacts with the introduced substance to neutralize its effects or to aid in the removal of the material from the body. The altered serum proteins are called antibodies and are found principally in the gamma globulin fraction. The introduced substances are called antigens. They are protein in chemical composition, although some polysaccharides and a few lipids are capable of stimulating antibody production.

The antigen-antibody reaction is a protective, defense mechanism when the antigen is an infectious agent. If the antigen is a foreign protein, the reaction causes alterations in the body response, harmful to the recipient, e.g., allergy. In some individuals normal constituents become altered in such a way that they act as antigens. This concept is known as auto-immunity, e.g., rheumatoid arthritis.

Serologic tests utilize the principles of agglutination, precipitation, and complement fixation. A brief resumè of each reaction follows.

A. AGGLUTINATION

This antigen-antibody reaction results in large aggregates of particles, which can be seen grossly or microscopically. Whole bacteria, whole red cells, or colloidal particles coated with antigen are the materials used. Serum specimens are serially diluted to give a semiquantitative estimate of the amount of antibody present.

B. PRECIPITATION

The antigen-antibody reaction gives a cloudiness to the mixture. The antigen is a soluble protein or in some instances, a polysaccharide. Serial dilutions of antigen are used instead of the usual serum (antibody) dilutions. The reaction is graded negative to plus-four, depending on the degree of resulting cloudiness.

C. COMPLEMENT-FIXATION

Antigen and antibody combine in the presence of and utilizing a serum component known as complement. This reaction produces either visible change, e.g., the lysis of bacteria or red cells, or no apparent change, in which case an

indicator system such as sensitized red cells is required. In the direct system the bacteria or red cells act as the antigen and are made susceptible to lysis by the antibody in the presence of complement. In the indirect system the antigen-antibody react in the presence of complement, removing it from the serum. Nothing is seen until the indicator system, consisting of previously sensitized red cells, is introduced. When antigen-antibody have reacted, no hemolysis is present. When no reaction has taken place, the indicator red cells are hemolyzed.

The introduction touches on the highlights of practical serologic tests and it gives the basis for their application to human disease. It is not complete for serology is a changing field, in which new procedures are rapidly developing. A list of some available reference works is included at the end of this section for those who wish a more complete understanding of basic serology.

D. GENERAL REFERENCES

Carpenter, P.L.: Immunology and Serology. 1956. W.B. Saunders Co., Philadelphia.

Dubos, R.J.: Bacterial and Mycotic Diseases of Man. 1959. J.B. Lippincott Co., Philadelphia.

Florey, H.: General Pathology. 1959. W.B. Saunders Co., Philadelphia.

Kahn, R.L.: Serology with Lipid Antigen. 1950. William & Wilins Co., Baltimore.

Kolmer, J.A.: Clinical Diagnosis by Laboratory Examination. 1961. Appleton-Century-Crofts, Inc., New York City.

Kolmer, J.A.; Spaulding, E.H. and Robinson, H.W.: Approved laboratory Technique. 1951. Appleton-Century-Crofts, Inc., N.Y. City.

Levinson, S.A. and MacFate, R.P.: Clinical Laboratory Diagnosis. 1961. Lea and Febiger, Philadelphia.

Manual: Serologic Tests for Syphilis. 1959. U.S. Department of Health, Education & Welfare, U.S. Public Health Service, Communicable Disease Center, Atlanta, Ga. (Venereal Disease Research Laboratory, Chamblee, Ga.)

Manual: Syphilis: Modern Diagnosis and Management. 1960. U.S. Department of Health, Education & Welfare, U.S. Public Health Service, Washington, D.C.

Miller. S.E.: Textbook of Clinical Pathology. 1960. Williams and Wilkins Co., Baltimore.

Perez-Tamayo: Mechanism of Disease: An Introduction to Pathology. 1961. W.B. Saunders Co., Philadelphia.

Rivers, T.M. and Horsfall, F.L., Jr.: Viral and Rickettsial Diseases of Man. 1959. J.B. Lippincott Co., Philadelphia.

SECTION II

SEROLOGIC SPECIMENS

A. TECHNIQUE OF VENIPUNCTURE

The technique of venipuncture can be readily demonstrated by a series of drawings as shown in the illustrations on page 7. The following may be of additional value to the student.

1. Equipment

Assemble equipment as listed below. Label tubes properly.

Cotton sponges, soaked with 70% alcohol.
Vacutainer tube for clotted blood.
Vacutainer adapter.
Disposable 21-gauge, 1 1/2-inch needles.
Tourniquet.
Paper clip or rubber band for attaching chit to tube.
Marking pencil to label tube.
Spirits of Ammonia.

a. All syringes, needles, lancets, or other instruments used for the collection of blood specimens must be sterile. Sterilization by dry heat or autoclaving is preferable and recommended, but boiling for a minimum of fifteen minutes is satisfactory. This is necessary to prevent the possible transmission of homologous serum jaundice.

2. Preparation

a. Place a tourniquet around the patient's arm above the elbow, tightly enough to check venous circulation but not so tightly as to stop arterial flow.

b. By inspection and palpation, locate the desired vein, determine the direction of its course, and estimate its size and depth.

c. Cleanse the skin over the selected vein. Place a sponge on the arm above the site of puncture. Allow the alcohol to dry before making the puncture to prevent tattooing the skin with alcohol. Do not contaminate the area after cleaning. (If blood is drawn for culture, use mild tincture of iodine or merthiolate. If blood is for a Bogen's test, use a nonalcoholic antiseptic, such as aqueous solution of merthiolate.)

d. Fit the needle to the syringe while the alcohol is drying. The plunger must match the syringe and must be held tightly in the cylinder at all times in order to prevent injection of air into the vein.

3. Procedure

a. Have the patient "make a fist" and straighten the arm. Frequently it is helpful to have the patient place the clenched fist of the other hand under the elbow to straighten the arm.

b. Grasp the syringe in the right hand (left for left-handed person) and place forefinger on the needle to guide it. With the needle pointing in the direction of the course of the vein, make the venipuncture. This operation may be considered in two steps: First the skin is broken; secondly, the vein is entered. A slight "give" indicates the vein is punctured. In vigorous individuals, blood will appear spontaneously. If pressure in the vein is low, the plunger may have to be withdrawn slightly to draw out the blood. Slide the needle a short distance (one-fourth to one-half inch) further into the vein to prevent the needle from coming out while the blood is being withdrawn. Take care not to go through the other side of the vein. Use the left hand to pull the plunger while steadying the syringe with the right hand.

c. Release the tourniquet. Place a sponge over the site of puncture and withdraw the needle. Instruct the patient to open the hand and press the sponge over the wound for 2 to 3 minutes. (For hematocrits and fragilities, the tourniquet is removed as soon as blood appears in the syringe.)

d. Remove the needle from the syringe and run blood down the side of the tube, using gentle pressure on the plunger to avoid foam and rupture of the cells. In filling the tubes with marked measurements, the needle is preferably left on and the plunger pulled back slightly just as the mark is reached to prevent more blood from entering the tube.

e. The oxalate and citrate containers for unclotted blood are filled before the tubes for clotted blood specimens.

f. The following points may be of help in difficult venipunctures:

(1) Veins may be made more prominent and easier to enter by allowing the arm to hang down for 2 to 3 minutes, by massaging toward the body, and by smartly but lightly slapping the site of puncture.

(2) In some persons veins become indistinct after the tourniquet has been on 2 or 4 minutes.

(3) Young and vigorous persons usually have elastic veins well filled with blood so that entrance to the vein is easy and the blood flow is good. Elderly or weak persons may have calcified veins which are hard to enter or of such low pressure that veins collapse under pressure of the needle.

(4) Occasionally the best vein is located in the hand, leg, or foot. These areas are more sensitive, however, and the veins usually less firmly anchored than those of the arm.

(5) Rolling veins may be held by placing the thumb on the vein so that a 1- or 2-inch length of vein lies between the thumb and the tourniquet. As soon as the vein is entered, the thumb is removed.

(6) Immersing the part in warm water or applying hot compresses to the area will cause veins to fill in some difficult cases.

(7) Be prepared to assist an individual who feels faint or actually becomes unconscious.

(a) Head between knees.

(b) Use of Spirits of Ammonia.

(c) If bed available, lay out flat, loosen clothing, and elevate legs slightly.

4. Obtaining a Clear Specimen

a. Draw before meals to avoid presence of chyle which appears in serum after a fatty meal.

b. Use disposable needles and sterile glassware.

c. Remove the needle and run the blood gently down the side of the tube to avoid rupture of cells.

d. Let stand at room temperature undisturbed for at least 30 minutes to clot.

e. Keep specimens in refrigerator until time for examination. Freezing will cause hemolysis.

f. If blood or serum is to be mailed:

(1) Collect under sterile conditions.

(2) Keep specimen and packing material in refrigerator until time for mailing.

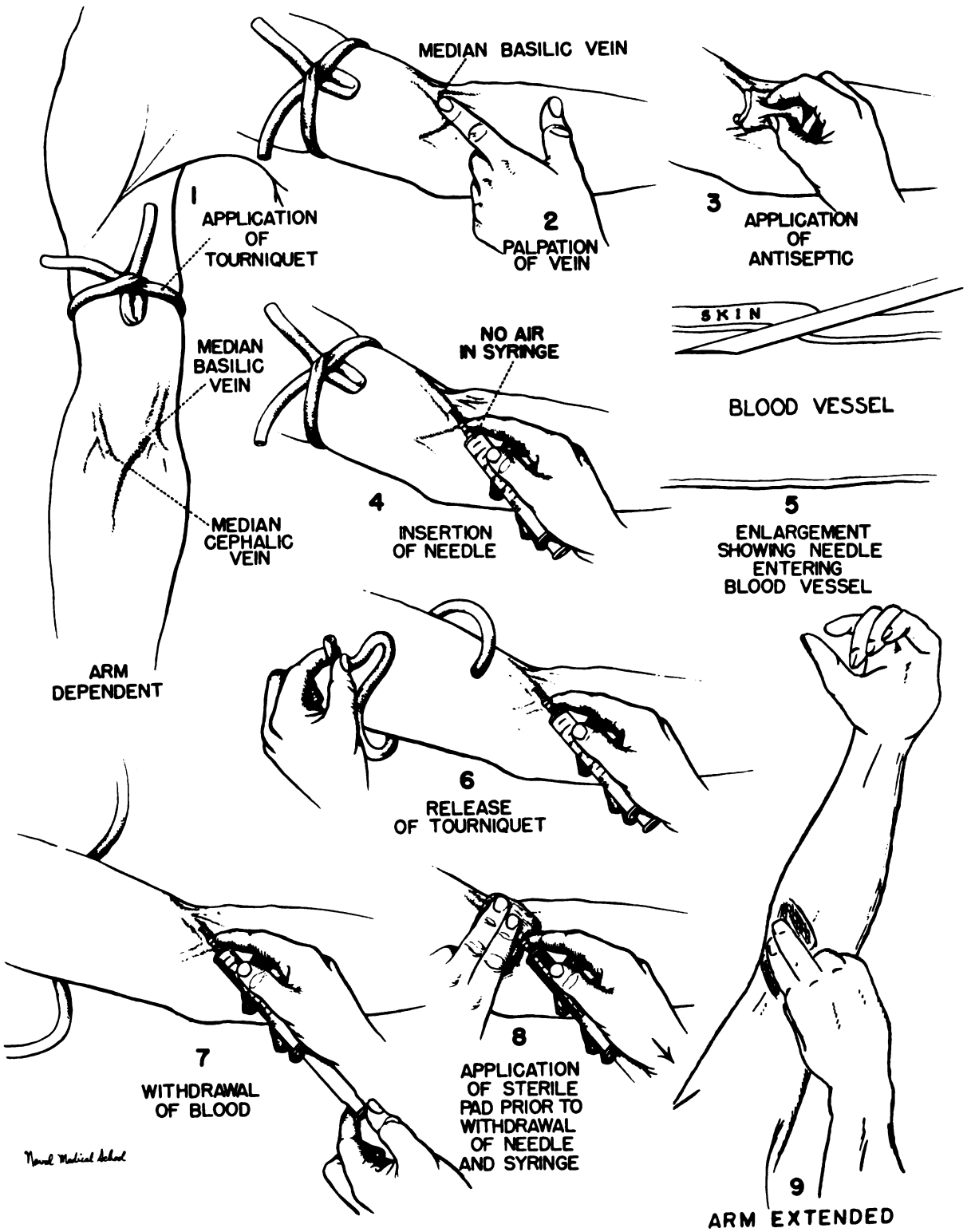
(3) Mail the same day as it is collected, because serum possesses antibacterial qualities only when fresh.

(4) If serum is to be mailed, separate from the clot under sterile conditions.

(5) Do not heat serum before mailing. Heat destroys its antibacterial properties.

g. Further instructions for the shipment of serums for routine serologic testing will be found under "B. Preparation for Shipment."

TECHNIQUE OF VENIPUNCTURE



B. PREPARATION FOR SHIPMENT

Each specimen should be accompanied by a Department of Defense Form No. 876, properly filled out and signed by proper authority.

Although spinal fluid and blood are usually drawn with reasonable attention to sterility, many specimens are contaminated when they are received in the laboratory.

1. Use of a Preservative

Spinal fluids and serums which have become grossly contaminated in transit are unsatisfactory for serologic tests for syphilis. Some of the products of bacterial metabolism are soluble and changes of the original components of the body fluids occur. Any serologic results obtained, therefore, are of questionable value.

a. Merthiolate: The use of merthiolate (sodium ethylmercurithiosalicylate)* as a bacteriostatic agent for spinal fluid has been reported. ** It does not interfere with the mechanisms of the serologic tests and does not introduce a dilution factor, except for TPI testing.

b. Preparation of Merthiolate Solution: On the day it is to be used, prepare the necessary amount of merthiolate solution by adding 1.0 gm. of merthiolate to each 100 ml. of distilled water. Do not use commercially prepared tinctures or solutions.

2. Preparation of Collection Tubes for Spinal Fluid for Routine Serology

a. Pipet 0.1 ml. of 1% aqueous merthiolate solution to the bottom of each 13 x 100 mm. tube.

b. Place the tubes in a vacuum desiccator over calcium chloride at room temperature, protected from the light. In 24 hours, dehydration is usually complete, if a satisfactory vacuum has been established.

c. For stoppers, submerge corks in hot (not smoking) paraffin for one minute and while hot, roll on a cloth to remove excess paraffin.

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* Federal Standard Stock Catalog item (10 gm. for preserving blood serum and vaccines).

** Harris, A. & Mahoney, J. F.: Merthiolate as an effective bacteriostatic agent in spinal fluid specimens. J. Ven. Dis. Inform., pp. 25, 46 (Feb 1944).

d. Remove the tubes from the desiccator and stopper tightly with paraffin corks.

e. Store the tubes in the dark. They will remain usable for months.

f. The concentration of merthiolate obtained when 2.0 ml. to 8.0 ml. of spinal fluid is added to each tube is sufficient to inhibit bacterial growth in transit.

3. Preparation of Collection Tubes for Serum for Routine Serology

Follow the same technique as for spinal fluid, except use 12 x 75 mm. tubes. One milligram of merthiolate is suitable for shipment of 2 ml. to 4 ml. of serum.

4. Preparation of Serum for TPI Test

a. All the equipment used to obtain the blood specimen must be chemically clean (free of acid and alkali) and sterile. See paragraph A-4 (Obtaining a Clear Specimen). A sterile paraffined cork should be used as a closure for the tube, or a sterile screw cap, because rubber contains a substance which is TOXIC to treponemes. Shipment in a vacutainer tube is harmful for the same reason.

b. A sample of 10 ml. of sterile serum should be sent at the earliest possible date, via air, whenever feasible. Label the tube "TPI SAMPLE." Include name, rate, and service number of patient. Label mailing carton "FOR TPI TEST."

c. Past experience has shown that if the donor has received injections of antibiotics, other than penicillin, within a month, or oral antibiotics within a week of drawing the blood sample, an inconclusive reaction will result. Aside from the foregoing, the following precautions in obtaining specimens will eliminate many unsatisfactory results:

(1) Glassware used in connection with obtaining and forwarding sera for TPI tests must be acid cleaned (potassium dichromate cleaning solution). After cleaning, each piece of glassware must be individually rinsed 15 times under running tap water and 10 times under running distilled water, then dried, plugged, and sterilized.

(2) Draw 20 cc of blood, allow to clot, then separate serum aseptically. Place serum in an acid cleaned sterile test tube and stopper with a paraffin coated cork. Uncoated cork stoppers or rubber stoppers are unsatisfactory. Test tube 16 x 125 mm. with a screw cap (Fed. Stand. Stock item) may also be used, or blood may be drawn in a vacuum tube, such as a B-D Vacutainer, providing the rubber stopper is immediately replaced with a coated cork stopper.

NOTE: 1. Vacutainers with special stoppers are available to use for TPI specimens.

2. Vacutainer with disposable needle and adapter are used in the same manner as needle and syringe in obtaining blood. This does not require extra steps and is the method of choice.

(3) It is highly recommended that a fasting blood sample be drawn, since the presence of post-prandial fats in the serum interferes with technical performance of the test.

(4) No chemical preservatives, such as merthiolate, sodium azide, etc., should be added to serum sample.

(5) Under no circumstances should whole blood be submitted for testing.

5. Preparation for Shipment of Darkfield Specimen

The specimen is collected in the manner as outlined under "Darkfield Examination" (Section III, paragraph A-1). The slide specimen, correctly labeled and accompanied by the Standard Form 514-C or 514-H, properly completed and signed, is placed in a slide box. Shipment is to the nearest hospital with facilities to do darkfield examination. The specimen will show viable organisms up to 48 hours after collection.

6. Special Precautions

Serum specimens must be without hemolysis, particularly for ASO titer. When hemolysis is present, a request for a repeat examination is usually made.

C. PREPARATION OF GLASSWARE

1. Introduction

All serological glassware which is to be used in the serodiagnostic laboratory must be chemically clean. Test tubes and pipet from which protein solutions have not been completely removed acquire a brownish film. This film can be removed by submersion in sulfuric acid - dichromate solution for 24 hours. Glassware which contains deposits of acid and/or alkali alter results with precipitin and agglutination tests. These materials destroy complement.

When either alkaline or acid solutions are used for cleaning dirty or stained glassware, the follow-up rinsing must be adequate to remove all cleaning solutions. It is recommended that all serological glassware be rinsed a minimum of 17 times in running tap water, followed by a minimum of five separate rinsings in distilled water.

A spot check of serological glassware is made daily. The chemical check is accomplished by employing indicator paper or indicator solution to insure against the release of chemically contaminated glassware. The cleanliness

check is made by filling and emptying a test tube or pipet. If beads of water adhere to the sides, the glassware is dirty and the cleaning process is repeated. Test tubes, slides, and pipets which become etched or scratched to such a degree that interference with test reading occurs, are discarded and replaced.

Various methods are employed for cleaning slides, pipets, and test tubes. An effective procedure for each is outlined below.

2. Special Cleaning Solutions

a. Sulfuric acid-dichromate:

(1) Formula - concentrated sulfuric acid ..... 2,000 ml.
water ..... 2,000 ml.
sodium or potassium dichromate .. 3 1/4 lbs.

(2) Procedure -

(a) Mix water and sodium dichromate until dissolved.

(b) Add sulfuric acid very slowly, stirring constantly, until the sodium dichromate reprecipitates.

NOTE: Great caution must be taken in adding the sulfuric acid to the sodium dichromate. The solution becomes intensely hot and may boil over. The transfer of cleaning solution from one container to another is carried out by a siphon arrangement as follows:

A rubber tube is attached to the suction arm of a water vacuum pump connected to a faucet. The far end of the rubber tube is lowered to the bottom of the container that is to be emptied of cleaning solution. By starting a stream of water through a faucet, a vacuum is created within the rubber tubing and the cleaning solution is immediately drawn into the tubing. By detaching this tubing from the side arm of the faucet and placing it in another container, the solution continues to flow into that container. A thin, acid-resistant rubber tubing is used for this purpose, making it possible to see the cleaning solution within the tube.

b. Aqua regia:

(1) Formula - Nitric acid (concentrated) .....1 part
Hydrochloric acid (concentrated) ....3 parts

NOTE: This potent mixture is kept under a hood with the additional precaution to keep the lid slightly ajar when the solution is first prepared. The potent fumes may blow off this lid. Prepare the solution in a glass container.

3. Procedure for Cleaning Glassware

a. VDRL Slides

(1) Procedure: The 2 x 3-inch flat slides which are used in the VDRL Test are cleaned with Bon Ami. Previously used paraffin-ringed slides are first freed of paraffin by passing them under hot running tap water. New and old slides are then washed with detergent followed by a two-minute rinse under running tap water. Then the slides are coated with Bon Ami and allowed to air-dry. When the slides are dry, the Bon Ami is removed with a soft cloth. The slides are ringed on a standard paraffin ringing machine which will deliver 12 rings, 14 mm. in diameter.

(2) Precautions: Slides are handled by the edges, to prevent greasy fingerprints on the testing surfaces. The failure of serum to spread evenly is an indication of an unclean slide. This slide is not used. Newer detergents require multiple rinsings, as traces of detergent sufficient to hemolyze red cells may be found after 17 rinses.

b. Pipets - 1.0, 5.0, 10.0 ml.

(1) Place pipets, points down, in plastic bucket with soapy water for one hour.

(2) Rinse pipets in an automatic rinser for 60 minutes.

NOTE: Adjust the flow so that the washer alternately fills and empties. If the flow of water is too slow, the washer fails to empty; if too fast, it will not fill.

(3) Drain the pipets, dry in a hot air oven, and permit to cool.

(4) If stained, placed in dichromate cleaning solution, preferably overnight. Drain off the bulk of the cleaning solution.

(5) Place pipets, points up, in the automatic pipet washer, taking care to remove any cleaning solution that has dripped onto the top of the washer or inside the washer at levels above the mark to which the water automatically rises. Such traces of cleaning solution will contaminate and nullify the effects of rinsing.

(6) Permit the pipets to rinse for an hour, checking to see that the washer is alternately filling and emptying.

(7) Place the pipets in distilled water for 20 minutes.

(8) Drain and dry in the oven.

NOTE: The inside of the pipet washer should be scrubbed and rinsed periodically.

c. Pipets - 0.1 and 0.2 ml.

These pipets have a very small bore, rendering the mechanical washer ineffective. Therefore, these soiled pipets should be placed in a cylinder of water. They should be cleaned by pulling tap water through them with the aid of a water suction pump, followed by distilled water. For rapid drying, one may follow the distilled water with a rinse of alcohol and then ether, still employing the suction pump to pull the fluids through the pipets.

4. Procedure for Washing Tubes

a. Clot tubes: Clots should be emptied into a wide-mouthed drain, followed by copious amounts of cold water. Tubes should be rinsed with water and placed in soap and water solution.

b. Serum tubes and other tubes (except Colloidal Gold Tubes)

(1) Empty contents of tubes in the sink.

(2) Submerge tubes in a bucket of soapy water.

(3) Wash each tube individually with a brush.

(4) Rinse tubes 17 times with running cold tap water.

(5) Rinse tubes 5 times in distilled water.

NOTE: Two buckets of distilled water may be used, rinsing tubes first in bucket #1 and then in bucket #2. Change the distilled water at least twice every day, and more often if the volume of glassware is great.

c. Colloidal Gold Tubes

(1) Empty contents of tubes in sink.

(2) Scrub tubes with soapy water. Use a brush and scrub each tube individually.

(3) Tubes are rinsed 17 times with running cold tap water and 5 times with distilled water.

(4) Tubes are dried and then placed in aqua regia overnight.

(5) Remove tubes from aqua regia.

(6) Each tube is rinsed 17 times directly under a stream of running tap water. Hold a few tubes in the hand and rinse under the faucet. Fill and empty tubes 17 times, and as the tube is inverted, rinse the outside.

(7) Rinse each tube individually 5 times with distilled water by holding the tubes under distilled water flowing from a bottle.

NOTE: All buckets used should be scrubbed, thoroughly rinsed, and turned over to drain at the end of each working day.

D. PREPARATION OF DILUTIONS

1. Introduction

Many laboratory procedures require that a substance under study be used in one or more concentrations less than that of the original material; with fluids, a convenient method of obtaining the desired conditions is to prepare dilutions that will contain decreasing amounts of the initial sample. A dilution is made by adding a known amount of the material concerned to a known amount of a suitable diluent, then mixing the two substances. It is customary to express the amount of material diluted in terms of the unit 1, which means 1 part of the substance contained in the total number of parts of the dilution. For example, if it is desired to test a material in one-tenth its original strength a dilution of 1 part in 10 may be made; each of the 10 parts of the dilution will then contain one-tenth (1/10 or 0.1) of the substance diluted. In this example, therefore, 1 part of the dilution would be used to determine the strength of one-tenth of the original substance. A dilution of 1 part in 10 is expressed by the symbol 1:10 and means that 1 part of the original material was mixed with 9 parts of diluent and so resulted in a total of 10 parts. Using serum as the material to be diluted, and physiologic saline solution as the diluent, the table below illustrates the preparation of some commonly used dilutions.

Ml. of serum		Ml. of saline		Total parts		Serum dilution
0.1	+	0.1	=	2	=	1:2
0.5	+	0.5	=	2	=	1:2
1.0	+	1.0	=	2	=	1:2
0.1	+	0.4	=	5	=	1:5
0.5	+	2.0	=	5	=	1:5
1.0	+	4.0	=	5	=	1:5
0.1	+	0.9	=	10	=	1:10
0.5	+	4.5	=	10	=	1:10
1.0	+	9.0	=	10	=	1:10
0.1	+	9.9	=	100	=	1:100
0.5	+	49.5	=	100	=	1:100
1.0	+	99.0	=	100	=	1:100

A higher dilution may readily be made from a lower dilution, some examples of which are shown below.

Serum dil. - ml. used			Ml. of saline	Calculation	Final Dilution
1:2	1.0	+	4.0	5 parts x 2	1:10
1:2	1.0	+	6.5	7.5 " x 2	1:15
1:10	1.0	+	4.0	5 " x 10	1:50
1:10	1.0	+	9.0	10 " x 10	1:100
1:100	6.0	+	4.0	1 2/3 " x 100	1:166
1:100	4.0	+	6.0	2.5 " x 100	1:250

NOTE: The higher dilution can be calculated by using a simple proportionality formula:

Serum dilution: Volume serum dil. used (amount) :: Final dilution (unknown) : Total final volume

Example:

Serum dilution is 1 : 2
Volume of serum dilution is 1.0 ml.
Total final volume is 5.0 ml.
Final dilution (unknown) is X
2 : 1.0 :: X : 5.0
10 = X

2. Serial Dilutions

In certain tests for serologic activity it may be desirable to titrate the serum in a series in which the dilution factor is constant; such a procedure is referred to as making serial dilutions of the serum. For example, if the dilution factor is 2, each dilution is twice that of the one immediately preceding it. Under these circumstances, it is usually desirable to plan mixtures of serum and diluent so that the final volume is the same in each tube; the description and table below illustrate one convenient means of preparing serial dilutions using 2 as the factor.

Ten 13 x 100 mm. test tubes are placed in a rack; the tenth tube is labeled C, for control, the others are numbered 1 through 9. Some means of identifying the serum is also placed on the label; for example, using serum A, the tubes would be marked A-1, A-2, etc., and the tenth tube A-C. Using a 5 ml. pipet, 0.5 ml. of physiologic saline solution is placed into each of the 10 tubes; then, using a sterile 1 ml. pipet, 0.5 ml. of the serum to be titrated is

removed from its container and mixed with the saline in tube 1. The same pipet may be used in making the succeeding dilutions in the series. The serum in tube 1 represents a dilution of 1:2 (0.5 ml. of whole serum + 0.5 ml. of saline, which is 1 part each of serum and diluent); remaining dilutions are then prepared as shown below.

	Ml. saline		Ml. serum or dilution	Calculation		Dilution
Tube 1	0.5	+	0.5 - whole serum	1 + 1	=	1:2
" 2	0.5	+	0.5 - from tube 1	2 x 2	=	1:4
" 3	0.5	+	0.5 - " " 2	2 x 4	=	1:8
" 4	0.5	+	0.5 - " " 3	2 x 8	=	1:16
" 5	0.5	+	0.5 - " " 4	2 x 16	=	1:32
" 6	0.5	+	0.5 - " " 5	2 x 32	=	1:64
" 7	0.5	+	0.5 - " " 6	2 x 64	=	1:128
" 8	0.5	+	0.5 - " " 7	2 x 128	=	1:256
" 9*	0.5	+	0.5 - " " 8	2 x 256	=	1:512
" 10	0.5	+	none	none		Control

* The procedure has left a volume of 0.5 ml. in each of the tubes except 9 which contains 1.0 ml.; accordingly, 0.5 ml. of this dilution (1:512) is discarded.

E. TYPES OF TUBES AND QUANTITIES OF SPECIMENS REQUIRED

The types of tubes and quantities of blood required for each serologic test are given below. The tubes used are Vacutainer tubes with a particular colored stopper.

1. VDRL	- 10 ml.)	
2. Kolmer	- 10 ml.)	
3. Reiter Protein	- 10 ml.)	
4. Mazzini	- 10 ml.)	
5. Davidsohn	- 10 ml.)	Clot tube (red stopper)
*6. Cold agglutinin	- 10 ml.)	
7. Ra Slide	- 10 ml.)	
8. Rose	- 10 ml.)	
9. L.E.	- 10 ml.)	
10. J.A.	- 10 ml.)	
**11. TPI	- 20 ml.	- Clot tube (yellow stopper)
12. FTA	- 10 ml.	- Clot tube (red stopper)
13. Spinal Fluids	See section pertaining to test	

*See special drawing instructions.

**This Vacutainer tube is a special clot tube for TPI specimens. The stopper is non-toxic and requires no further processing.

SECTION III

SERODIAGNOSIS OF SYPHILIS

A. INTRODUCTION

Syphilis is a specific infectious disease, caused by the spirochete, Treponema pallidum. It is characterized by widespread involvement of the tissues with protean manifestations. The usual mode of transmission is sexual contact. The diagnosis is established by history of contact, by physical examination, by detection of the organism using darkfield preparations, and by a combination of available serologic tests. The course of syphilis is conveniently divided into stages: primary, secondary, latent, and tertiary (late manifestations). Syphilis acquired in utero causes a congenital form. The treatment of all forms is adequate amounts of penicillin.

The two principal laboratory methods of diagnosis are darkfield preparation and serologic tests.

1. Darkfield examination

With a darkfield microscope, or with an ordinary microscope and darkfield condenser, the morphology of living spirochetal organisms is demonstrated. This technique is excellent if properly used. The living organisms are found in the lesions of primary and secondary syphilis. Darkfield positive results give an absolute diagnosis.

NOTE: Darkfield technique shows the presence of different types of spirochetes, but experience helps in differentiating the organisms. Negative results mean the lesion is not syphilitic, the therapy has killed the organism, or the lesion is in the healing stage.

2. Serologic tests

Serologic tests detect the presence of antibody which has developed in response to the organism. The presence of detectable antibody is first noted one to three weeks after the development of the primary lesion. All serologic tests are felt to be positive by the secondary stage. The antibodies reach their peak during the secondary stage and tend to fall to lower levels. Occasional individuals become sero-negative. The antibodies fall into two types: reagin and treponemal.

a. Reagin is a non-specific antibody complex. It is measured by non-treponemal antigen obtained by extracting beef heart. Two types of non-treponemal tests are used: flocculation, e.g., VDRL, Mazzini; complement fixation, e.g., Kolmer. These are not the only tests which have been developed,

*Venereal Disease Research Laboratory

but are representative of the group as a whole. These tests vary in sensitivity and specificity, but they show good correlation with clinical disease.

b. Tests utilizing treponemal antigens are specific, however they are variable in sensitivity. They are expensive and cumbersome to perform. Their application is limited to confirming problem cases. Several varieties of treponemal antigens are available and the following chart summarizes the antigens and the tests.

I. Whole Body (all use Nichol strain treponeme)
   A. Viable treponeme
      1. Treponema pallidum immobilization (TPI)
   B. Non-viable
      1. Treponema pallidum agglutination (TPA)
      2. Fluorescent treponemal antibody (FTA)

II. Fractional Treponemal Antigen
   A. Kolmer test with Reiter protein antigen

3. Practical use of serologic tests

Because of the multiplicity of serologic tests for syphilis, it is necessary to select the ones most suitable for use in the particular laboratory concerned. The following procedures are used at this facility.

a. Screening test: VDRL Qualitative Slide Test is used to screen all serums. This procedure is used because of its sensitivity. When the VDRL Qualitative Slide Test is negative, no further studies are performed.

b. STS Battery: This includes the Quantitative VDRL Tube Test and the Quantitative Kolmer Complement Fixation Test. The battery is noted to include two types of reagin. This series of tests is used when the VDRL Qualitative Slide Test is weakly reactive or reactive.

c. Treponema pallidum Immobilization: When the STS Battery is reactive or when equivocal results are obtained, a request for serum to perform the TPI and to repeat the STS Battery is submitted.

B. DARKFIELD EXAMINATION

1. Introduction and Purpose of Darkfield Examination

The darkfield examination is conducted to aid the medical officer in the diagnosis and differential diagnosis of lesions suspect for syphilis. Material from primary and secondary lesions of the genital area, lesions occurring at

other sites, and material obtained by aspiration of regional lymph nodes are positive for T. pallidum in a large portion of cases. A positive examination is diagnostic of primary or secondary syphilis. Three negative examinations at daily intervals are required before the report is sent as negative. These are entrusted only to those who have adequate skill and experience to avoid an error of mistaking non-pathogenic treponemes for T. pallidum. When healing of the suspect lesion occurs, darkfield examinations are not applicable and yield negative results. Negative results are largely due to difficulty in securing the specimen material.

2. Equipment for the performance of the Darkfield Examination

a. Microscope with funnel stop for oil immersion
b. Darkfield condensor
c. Microscope lamp
d. Syringe, 20 ml., with the head removed and the plunger inserted at the reverse end. (This syringe would be set just the reverse of a normal-appearing one.)
e. Bacteriological loop
f. Gauze
g. Absorbent cotton
h. Microscopic slides and cover slips

3. Reagents for the performance of the Darkfield Examination

a. Saline, 0.9%
b. Petroleum jelly

4. Preparation of Equipment

a. The darkfield system: In this procedure, the object being examined is illuminated in such a way that no direct light reaches the microscope objective, instead only that light which is refracted, diffracted, or scattered by the object reaches the objective lens. Thus, the object being examined is seen as a bright image on a dark background. In general, there are three principal methods employed in darkfield microscopy which are dependent upon the type of condenser used, as follows:

(1) Abbe's condenser with dark ground stop, with which only the low-power objective can be used and for which a special light source is required.

(2) Cardioid condenser, which requires a special light source and special glass (quartz) slides on which the specimens to be examined are placed.

(3) Paraboloid condenser, with which the usual microscope lamp can be used and the high-power objective also can be employed. Since paraboloid condensers are generally available and satisfactory, the darkfield system and

procedures described below will be based upon this type of equipment.

b. Prior to the actual performance of the darkfield examination the microscope is set up with the proper darkfield condenser and the ring on the surface of this condenser must appear in the center of the field when viewing the condenser through the low-power objective of the microscope. Focus the ring into the field and if it is not centered, make the proper adjustment of the ring by adjusting the set screw which appear just below the stage of the microscope and on the front of the darkfield condenser. The ring on the darkfield condenser is centered to allow the maximum amount of light to pass through the condenser to allow the technician to study the slide with the greatest illumination possible.

c. Place a drop of immersion oil on the upper surface of the condenser then, by means of the condenser adjustment knob, lower the condenser about 1/4 to 1/2 inch; next, secure the slide preparation to be examined between the arms of the mechanical stage and center the area to be studied over the aperture in the microscope stage.

d. By means of the condenser adjustment knob, carefully raise the condenser until the oil on the condenser makes contact with the under surface of the object slide which will result in the area of the slide just above the condenser becoming quite luminous.

e. Still using the low-power objective, focus on the object slide and adjust the condenser until the smallest disc of light possible is obtained. Next, the higher power objectives may be used, as follows:

(1) The high-dry objective (4 mm.) should always be used to study characteristic motility of spirochetes such as T. pallidum; moderately large, actively motile organisms tend to "dive" out of the microscopic field.

(2) The oil immersion objective (1.9 mm.) is employed for detailed study of the finer morphologic structures of spirochetes when their motility has decreased and, when this lens is used, a funnel stop must be inserted into the objective.

(3) The purpose of the funnel stop is to prevent direct rays of light from entering the margin of the lens. The funnel stop is installed by unscrewing the oil immersion objective from the nosepiece, then inserting the funnel stop into the objective with the apex toward the lens, and replacing the objective in the microscope nosepiece. After placing a drop of immersion oil on the object slide, the oil immersion objective is focused on the object as described in subparagraph (e) above.

f. Important points to check in the darkfield system include:

(1) Be sure that all lenses are clean and free from dust and lint.
NOTE: Camel's-hair brush is often utilized to clean lens.

(2) Avoid air bubbles in the preparation to be studied and in the immersion oil used on the object slide and the condenser lens.

(3) Make certain that the funnel stop is correctly installed.

(4) The glass slide and cover slip used must be clean, free from dust, lint, and scratches.

(5) Use extreme care in focusing to avoid scratching the condenser and objective lenses; there should be enough immersion oil on the upper surface of the condenser to serve as a cushion between the lens and the object slide.

(6) It must be remembered that darkfield illumination requires an intense light.

5. Procedure for Darkfield Examination

a. The lesion should be cleaned and abraded with gauze or absorbent cotton to produce superficial bleeding. This is best accomplished by giving the patient a beaker of saline and asking him to wash and clean the area with a continuous soaking for at least 15 minutes. The lesion is then dried and the heel of the barrel of a 20 ml. syringe is placed over the lesion and the plunger is withdrawn to create a suction to the infected part thereby drawing serum through the lesion and forcing the spirochetes to the surface. Remove all initial blood and allow the serum to ooze from the site. A drop of this serum is then picked up with a bacteriological loop and placed in a drop of normal saline which was previously placed on a clean glass microscopic slide. After completion of this step a clean cover slip is placed over the serum-saline combination. Place a drop of immersion oil on the darkfield condenser. Place the slide on the microscope stage and lift the darkfield condenser until the oil which is resting on apex (upper surface) of the condenser touches the bottom surface of the slide containing the specimen for study. Now change from the low power objective to the high power objective and lower the barrel of the microscope until it is approximately 5 mm. from the surface of the slide. Now using the fine adjustor of the microscope lower the objective until the specimen is brought into the field.

b. Using the high dry objective, the film of exudate is carefully and systematically observed for the presence of typical spirochetes; the organisms should appear as bright objects against a black background and, in fresh preparations, are actively motile. Obtain as much information as possible concerning the morphologic characteristics of the organisms by careful study of individual cells.

c. For more detailed study of the finer structures of the spirochetes,

the oil immersion objective is used after first installing the funnel stop; this lens is of greatest value after the cells have lost some of their motility.

NOTE: Each specimen should be studied for at least 30 minutes to enable the technician to completely cover the surface of the slide. One spirochete of T. pallidum is sufficient to diagnose primary or secondary syphilis.

6. Summary and Sources of Error

Failure to demonstrate the organism from a suspected syphilitic lesion by darkfield examination may mean:

a. The lesion is not syphilitic.

b. The patient has received antisyphilitic drugs locally or systemically.

c. Too much time has elapsed since the appearance of the lesion.

d. Inexperience in technique.

Practice is required in the proper collection of material to be examined, as well as in the identification of the organism seen. T. pallidum and various nonpathogenic organisms appear morphologically identical, and may be easily confused.

The expert can readily distinguish T. pallidum from the usual nonpathogenic spirochetes occurring in genital or skin lesions. Normal appearing spirochetes from the mouth, especially around the gums, however, may be confused with T. pallidum and suspected syphilitic lesions from this area should only be performed by experts.

NOTE: In the event of a delay from the time the specimen is collected and the time of the actual performance of the test, rim the edges of a coverslip with a small amount of petroleum jelly as a seal to prevent escape of infectious material and to delay evaporation of the specimen. A specimen prepared in this manner can be read up to a period of four hours.

C. VDRL FLOCCULATION TESTS WITH SERUM

1. Materials and Preparations for Slide Tests - Serum

a. Equipment:

(1) Rotating machine, adjustable to 180 rpm, circumscribing a circle 3/4 inch in diameter on a horizontal plane.

(2) Ringmaker, to make paraffin rings approximately 14 mm. in diameter.

(3) Slide holder, for 2 x 3 inch microscope slides.

(4) Hypodermic needles, of appropriate sizes, with or without points.

(a) 18-gauge without bevel to deliver 1/60ml/drop. To be used in Qualitative Slide Test.

(b) 19-gauge without bevel to deliver 1/75ml/drop. To be used in Quantitative Slide Test.

(c) 23-gauge with bevel to be used in Quantitative Slide Test.

b. Glassware:

(1) Slides, 2 x 3 inch, with paraffin rings approximately 14 mm. in diameter.

NOTE: Glass slides, microscopic, 12 concavities, Kline, agglutination, 2cm. x 2 1/4 in. x 3mm. (Source: Scientific Products - Stock #M6200.)

(2) Bottle, 1 oz., Pyrex, flat-bottomed, glass-stoppered. (These are available from Corning Glassware, Catalogue CA-1 90530.)

(3) Syringe, tuberculin type.

c. Reagents:

(1) Antigen:

Antigen for this test is an alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol, and sufficient purified lecithin to produce standard reactivity. During recent years this amount of lecithin has been 0.21% ± 0.01%.

(a) Each lot of antigen must be serologically standardized by proper comparison with an antigen of known reactivity.

(b) Antigen is dispensed in hermetically sealed glass ampules, and stored at room temperature (73° to 85°F.).

(c) The components of this antigen remain in solution at normal temperature so any precipitate noted will indicate changes due to factors such as evaporation or additive materials contributed by pipets. Antigen containing precipitate should be discarded.

(2) Saline solutions

(a) Buffered saline solution containing 1% sodium chloride:

Formaldehyde, neutral, reagent grade, ml.	0.5
Secondary sodium phosphate (Na_2HPO_4 + 12 H_2O), gm.	0.093
Primary potassium phosphate (KH_2PO_4), gm.	0.170
Sodium chloride (A.C.S.), gm.	10.0
Distilled water, ml.	1,000.0

This solution yields potentiometer readings of pH 6.0 + 0.1 and is stored in screw-capped or glass-stoppered bottles.

(b) 0.9% saline solution:

Add 900 mg. of dry sodium chloride to each 100 ml. of distilled water.

d. Preparation of Serum

(1) Clear serum, obtained from centrifuged, clotted blood, is heated in a 56°C. water bath for 30 minutes before being tested to inhibit action of complement.

(2) All serums are examined when removed from the water bath and those found to contain particulate debris are recentrifuged.

(3) Serums to be tested more than 4 hours after the original heating period should be reheated at 56°C. for 10 minutes.

e. Preparation of Slides

(1) New slides are cleaned with Bon Ami which is removed with a soft cloth after drying.

(2) Previously used slides are first freed of paraffin, washed with detergent, rinsed free of cleaning compound, and then treated as new slides.

(3) Slides are handled by the edges, while cleaning, to prevent greasy fingerprints on the testing surfaces.

(4) Serums will spread within the circles on clean slides. Failure of the serums to spread is an indication that the slide is unclean and therefore should not be used.

(5) Paraffin rings are made by transferring heated paraffin to the slides by means of metal molds.

f. Preparation of Antigen Emulsion

(1) Pipet 0.4 ml. of buffered saline solution to the bottom of a 1 ounce pyrex, flat-bottomed, glass-stoppered bottle.

(2) Add 0.5 ml. of antigen (from the lower half of a 1.0 ml. pipet graduated to the tip) directly onto the saline solution while continuously rotating the bottle on a flat surface.

NOTE: Antigen is added drop by drop, but rapidly, so that approximately 6 seconds are allowed for each 0.5 ml. of antigen. Pipet tip should remain in upper third of bottle and rotation should not be vigorous enough to splash saline solution on the pipet. Proper speed of rotation is obtained when the outer edge of the bottle circumscribes a 2 inch diameter circle approximately three times per second.

(3) Temperature of buffered saline solution and antigen should be in the range of 23° to 29°C. at time antigen emulsion is prepared.

(4) Blow last drop of antigen from pipet without touching pipet to saline solution.

(5) Continue rotation of bottle for 10 seconds more.

(6) Add 4.1 ml. of buffered saline solution from 5 ml. pipet.

(7) Place top on bottle and shake vigorously for approximately 10 seconds.

(8) Antigen emulsion is then ready for use and may be used during 1 working day (24 hours).

(9) Double this amount of antigen emulsion may be prepared at one time by using doubled quantities of antigen and saline solution. A 10 ml. pipet should then be used for delivering the 8.2 ml. volume of saline solution. If larger quantities of antigen emulsion are required, more than one mixture should be prepared. These aliquots may then be tested and pooled.

g. Testing Antigen-Emulsion Delivery Needles

The number of antigen particles per microscopic field is determined by the size of antigen-emulsion drop used. For this reason the needle used each day should be checked.

(1) Antigen emulsion is dispensed from a syringe fitted with an 18-gauge needle, without bevel.

NOTE: It is of primary importance that the proper amount (1/60ml) of antigen emulsion be used in each qualitative test, so this method of delivery, which will produce drops of constant and proper size, is recommended. See Fig. 1.

(2) If the four/fifths volume quantitative VDRL slide method is employed, a smaller gauge needle (without bevel) that will deliver 75 drops of antigen emulsion per milliliter must be employed for this procedure. A 19-gauge needle, without bevel, is used. Practice will allow rapid delivery of antigen emulsion, but care should be exercised to obtain drops of constant size.

(3) When allowed to stand, antigen emulsion should be gently mixed before use by rotating the bottle and emptying and refilling the syringe.

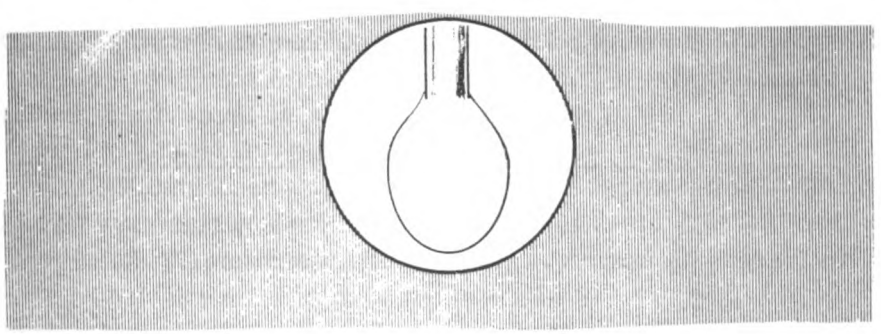


Fig. 1.

h. Preliminary Testing of Antigen Emulsion

(1) Each preparation of antigen emulsion should first be examined by testing serums of known reactivity in the Reactive, Weakly Reactive, and Non-reactive zones. This is accomplished by the method described under "VDRL

Slide Qualitative Test with Serum". These tests should present typical results and the size and number of antigen particles in the Nonreactive serum should be optimum.

(2) Only those antigen emulsions that have produced the designated reactions in tests performed with control serums (Reactive, Weakly Reactive, and Nonreactive) should be used. If antigen particles in the Nonreactive serum tests are too large, the fault may be in the manner of preparing antigen emulsion, although other factors may be responsible.

(3) An unsatisfactory antigen emulsion should not be used.

2. VDRL Qualitative Slide Test - Serum

a. Procedure:

(1) Pipet 0.05 ml. of inactivated serum, cooled for 10 min., into one ring of a paraffin-ringed glass slide.

(2) While holding syringe vertically with attached 18-gauge needle without bevel, add one drop (1/60ml) of antigen emulsion onto each serum.

(3) Rotate slides for 4 minutes. (Mechanical rotators that circumscribe a 3/4 inch diameter circle should be set at 180 rpm. Rotation by hand should circumscribe a 2-inch diameter circle 120 times per minute.)

(4) Read tests immediately after rotation.

NOTE: Serum controls of graded reactivity (reactive, weakly reactive, and non-reactive) are always tested prior to running a series of tests.

b. Reading and Reporting Qualitative Slide Tests Results - Serum

(1) Read tests microscopically, with low power objective, at 100 x magnification. The antigen particles appear as short rod forms at this magnification.

Aggregation of these particles into large or small clumps is interpreted as degrees of reactivity.

Reading	Report
No clumping or very slight roughness	Nonreactive (N).
Small clumps	Weakly Reactive (WR)
Medium and large clumps	Reactive (R)

(2) Zonal reactions, due to an excess of Reactive serum component, are recognized by irregular clumping and the loosely bound characteristics of the clumps. The usual Reactive finding is characterized by large or small clumps of fairly uniform size. Practical evidence will allow differentiation to be made between this type of reaction and the zonal picture wherein large and/or small clumps may be intermingled with free antigen particles. A zonal reaction is reported as Reactive. In some instances, this zoning effect may be so pronounced that a Weakly Reactive result is produced by a very strongly

Reactive serum. It is therefore recommended that all serums producing Weakly Reactive results in the qualitative test be retested using the quantitative procedure before a report of the VDRL slide test is submitted. When a Reactive result is obtained on some dilution of a serum that produced only a Weakly Reactive result as undiluted serum, the report is Reactive (see "Reading and Reporting Quantitative Slide Test Results," under "VDRL Quantitative Slide Tests with Serum").

3. VDRL Quantitative Slide Test - Serum

All serums that produce Reactive or Weakly Reactive results in the qualitative VDRL slide test should be quantitatively retested by the Quantitative Slide Test. Since this procedure, in most instances, provides for direct measurements of serum and saline solution, this method is efficient in its requirement of technician-time and amount of glassware employed.

a. Procedure:

(1) Place four 2 x 3 inch glass slides with 12 paraffin rings in a 5 place holder. NOTE: The holder accommodates up to 8 test sera.

(2) Prepare a 1:8 dilution of each serum by adding 0.1 ml. of the inactivated serum to 0.7 ml. of the 0.9% saline solution using a 0.2 ml. pipet graduated in 0.01 ml.

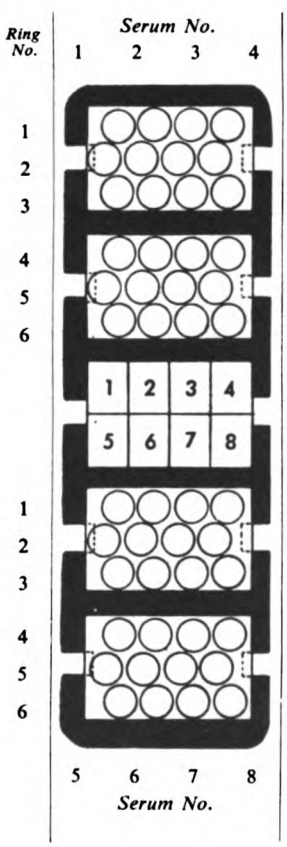
(3) Mix the serum and saline solution thoroughly and then allow the pipet to stand in the test tube.

(4) Using this pipet, transfer 0.04 ml., 0.02 ml., and 0.01 ml. quantities of the 1:8 serum dilution into the fourth, fifth, and sixth paraffin rings, respectively.

(5) With the same pipet, transfer 0.04 ml., 0.02 ml., and 0.01 ml. of the undiluted serum into the first, second, and third paraffin rings, respectively.

(6) Repeat this procedure with each serum and the accompanying 1:8 serum dilution until each of the eight serums are pipetted into their respectively numbered places on the slides.

Table 1.



Quantitative Slide Test		
Serum (ml.)	*Saline solution (ml.)*	*Serum dilutions*
0.04	0	1:1 (undiluted)
.02	.02	1:2
.01 (diluted 1:8)	.03	1:4
.04	0	1:8
.02	.02	1:16
.01	.03	1:32
.04	0	1:1 (undiluted)
.02	.02	1:2
.01 (diluted 1:8)	.03	1:4
.04	0	1:8
.02	.02	1:16
.01	.03	1:32

(7) Add 2 drops (0.01 ml. in each drop) of 0.9% saline solution to the second and fifth rings of each serum, by vertical delivery from a 23-gauge hypodermic needle fitted to a glass syringe.

NOTE: Needle should be checked for proper drop size. Saline

solutions will be delivered from a 23-gauge needle (0.01 ml. per drop).

(8) Add three drops of 0.9% saline solution (delivered in the same manner) of the same size to the third and sixth rings of each serum.

(9) Rotate slides gently by hand for about 15 seconds at 120 rpm to mix the serum and saline solution.

(10) Add one drop (1/75 ml.) of antigen emulsion to each ring using a syringe and needle of appropriate size. (Caution. Note that the amount of antigen emulsion used in this method has been reduced to 1/75 ml. to correspond with the reduced serum volume of 0.04 ml.)

(11) Complete tests in the manner described for the "VDRL Qualitative Test with Serum" (p. 26) and read results microscopically immediately after rotation. By this method, the dilutions of each serum are 1:1 (undiluted), 1:2, 1:4, 1:8, 1:16, and 1:32.

(12) If all serum dilutions tested produce Reactive results, prepare a 1:64 dilution of that serum in saline solution. Add seven parts of saline solution to one part of the 1:8 serum dilution, and test in three amounts as was done with the 1:8 serum dilutions. Dilutions prepared from the 1:64 dilution will be equivalent to 1:64, 1:128, and 1:256.

b. Reading and Reporting Quantitative Slide Test Results - Serum

(1) Read tests microscopically at 100 x magnification as described for qualitative procedure.

(2) Report results in terms of the greatest serum dilution that produces a Reactive (not Weakly Reactive) result in accordance with the following examples:

Table 2. Reporting Results.

Undiluted serum	Serum dilutions				Report
1:1	1:2	1:4	1:8	1:16	
R	WR	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	WR	N	N	Reactive, 1:2 dilution, or 2 dils.
R	R	R	WR	N	Reactive, 1:4 dilution, or 4 dils.

NOTE: Under conditions of high temperature and low humidity which are sometimes present during the summer months in certain areas, antigen emulsion may be stored in the refrigerator but should be restored to room temperature before use. To avoid surface drying under these conditions, tests should be completed and read as rapidly as possible. Slide covers containing a moistened blotter may be employed.

4. Materials and Preparations for Tube Tests - Serum

a. Equipment:

Equipment is same as used in Slide Test (see Equipment, paragraph C-1-a, Materials etc. - Serum, p. 22, except:

(1) Kahn shaking machine (must be operated at 275 to 285 oscillations per minute.)

(2) Microscope lamp.

(3) Microscope mirror.

b. Reagents:

(1) Antigen (same as for VDRL Slide Test).

(2) Saline solutions:

(a) Buffered saline solution, 1% (same as for Slide Test).

(b) Unbuffered, 1%, sodium chloride solution.

Add 1 Gm. of dry sodium chloride (chemically pure reagent grade) to each 100 ml. of distilled water.

c. Preparation of Serum:

(1) Serum, void of cells, is heated in a 56°C. water bath for 30 minutes before being tested, to inhibit complement.

(2) All serums are examined when removed from the water bath and those found to contain particulate debris are recentrifuged.

(3) Serums to be tested more than 4 hours after being heated should be reheated at 56°C. for 10 minutes.

d. Preparation of Antigen Emulsion:

(1) Prepare antigen emulsion as described for the VDRL Slide Test (Para.f, p.24).

(2) Add four parts of 1%, unbuffered sodium chloride solution to one part of VDRL Slide Test emulsion. Mix well and allow to stand five minutes before use. (Antigen must be used within two hours.) This solution will be referred to as "diluted antigen emulsion." Resuspend diluted antigen emulsion before use.

5. VDRL Qualitative Tube Test - Serum

a. Procedure:

(1) Pipet 0.5 ml. of heated serum into a 12 x 75 mm. (outside dimension) test tube.

(2) Add 0.5 ml. of diluted antigen emulsion to each serum.

(3) Shake tubes on Kahn shaker for 5 minutes.

(4) Centrifuge all tubes for 10 minutes at force equivalent to 2,000 rpm in No. 1, or 1,700 rpm in No. 2, I.E.C. (International Equipment Co., Boston, Mass.) centrifuge with horizontal heads.

(5) Return tubes to the Kahn shaking machine and shake for exactly 1 min.

NOTE: Include reactive and non-reactive control serums in each test run.

b. Reading and Reporting Qualitative Tube Test - Serum

(1) Read test results as soon as secondary shaking period is completed by holding tubes between rays of microscope lamp and microscope mirror.

(2) Record results as follows:

Reactive..... Visible aggregates in a clear or slightly turbid medium
All borderline reactions, where the observer has doubt regarding visible clumping, should be reported as "non-reactive."

Non-reactive..... No visible clumping or aggregation of antigen particles.
Appearance slightly turbid or granular.
Definite silken swirl on gentle shaking.

NOTE: Chylous or hemolyzed serums may cause completed tests too turbid for macroscopic reading and are therefore unsatisfactory specimens for this test.

(3) Zonal reactions, due to excess of reactive serum component, may appear to be very weak or, in rare instances, non-reactive. Whenever a zonal reaction is suspected, another test should be performed, using 0.1 ml. of heated serum and 0.4 ml. of saline solution in place of the original 0.5 ml. of serum. If a reactive finding is obtained with the smaller quantity of serum, a Quantitative Tube Test should be perofrmed.

6. VDRL Quantitative Tube Test - Serum

a. Procedure:

(1) Pipet 0.5 ml. of freshly prepared 0.9% saline solution into each of five or more test tubes (12 x 75 mm.), omitting the first tube.

(2) Add 0.5 ml. of heated serum to the first and second tubes. (The first tube may be omitted if the VDRL Qualitative Tube Test has been performed, and if sufficient serum is not available.)

(3) Mix and transfer 0.5 ml. from second to third tube.

(4) Continue mixing and transferring 0.5 ml. from each tube to the next until the last tube is reached.

(5) Mix and discard 0.5 ml. from last tube.

(6) Add 0.5 ml. of diluted antigen emulsion to each tube and proceed as described under VDRL Qualitative Tube Test with Serum.

b. Reading and Reporting Quantitative Tube Test Results - Serum

The greatest serum dilution producing a definitely reactive result is reported as the reactivity end point as shown in the following examples:

Table 3. Reporting Results

Undiluted serum	Serum Dilutions				Report
1:1	1:2	1:4	1:8	1:16	
R	N	N	N	N	Reactive, undiluted only, or 1 dil.
R	R	R	N	N	Reactive, 1:4 dilution, or 4 dils.
R	R	R	R	N	Reactive, 1:8 dilution, or 8 dils.

R = Reactive N = Non-reactive

D. VDRL FLOCCULATION TESTS WITH SPINAL FLUID

1. Materials and Preparations for VDRL Tube Tests - Spinal Fluid

a. Equipment:

Kahn shaking machine
(Must be operated at 275 or 285 oscillations/minute)

b. Reagents:

(1) Antigen (same as for VDRL Slide Test antigen - see p. 23).
(2) Saline solutions:
(a) Buffered, 1% saline solution
(Prepare as for VDRL Slide Test - see p. 23).
(b) Sodium Chloride solution, 10%.
Dissolve 10 Gm. of dry sodium chloride (chemically pure reagent grade) in 100 ml. of distilled water.

c. Preparation of Spinal Fluid:

(1) Centrifuge and decant each spinal fluid.

Spinal fluids which are visibly contaminated or contain gross blood are unsatisfactory for testing.

(2) Heat spinal fluid at 56°C. for 15 minutes. Cool to room temperature before testing.

d. Preparation of the Sensitized Antigen Emulsion:

(1) Prepare antigen emulsion as described for the VDRL Slide Test (see p. 24).

(2) Add one part of 10% sodium chloride solution to one part of VDRL Slide Test emulsion.

(3) Mix well, allow to stand at least 5 minutes, but not more than two hours before use.

2. VDRL Qualitative Tube Test - Spinal Fluid

a. Procedure:

(1) Pipet 1.0 ml. of heated spinal fluid into a 13 x 100 mm. test tube. Include reactive and non-reactive spinal fluid controls with each series of tests run.

(2) Add 0.2 ml. of sensitized antigen emulsion to each spinal fluid. Resuspend the sensitized antigen emulsion immediately before use by inverting container several times.

(3) Shake racks of tubes on Kahn shaking machine for 15 minutes.

(4) Centrifuge all tubes for 5 minutes at force equivalent to 1,800 rpm.

(5) Return tubes to Kahn shaking machine and shake exactly 2 minutes.

b. Reading and Reporting Qualitative Tube Test Results - Spinal Fluid:

(1) Read test results as soon as possible after the secondary shaking period, by holding tubes close to the shade of a desk lamp having a black background.

NOTE: Each tube may be held motionless or shaken gently during the reading. Excessive agitation should be avoided.

(2) Report results as follows:

Reactive..... Definitely visible aggregates suspended in a water-clear or turbid medium.

Non-reactive ... No aggregation, complete dispersion of particles, appearance turbid or slightly granular.

3. VDRL Quantitative Tube Test - Spinal Fluid

Quantitative tests are performed on all spinal fluids found to be Reactive in the qualitative test.

a. Procedure:

(1) Prepare spinal fluid dilutions as follows:

(a) Pipet 1.0 ml. of 0.9% sodium chloride solution into each of five or more tubes.

(b) Add 1.0 ml. of inactivated spinal fluid to tube 1, mix well, and transfer 1.0 ml. to tube 2.

(c) Continue mixing and transferring from one tube to the next until the last tube contains 2 ml. Discard 1.0 ml. from the last tube. The respective dilution ratios are 1:2, 1:4, 1:8, 1:16, 1:32, etc.

(2) Test each spinal fluid dilution as described under VDRL Qualitative Tube Test with Spinal Fluid, paragraph 2 above.

b. Reading and Reporting Quantitative Tube Test Results - Spinal Fluid

(1) Read each tube as described under VDRL Qualitative Tube Test with Spinal Fluid.

(2) Report test results in terms of the highest dilution of spinal fluid producing a Reactive result. The term "dils" which expresses the same dilution reactivity end point may be applied.

Example:

Table 4. Reporting Results

Spinal fluid dilutions					Report
1:2	1:4	1:8	1:16	1:32	
N	N	N	N	N	Non-reactive[1]
R	R	R	N	N	Reactive, 1:8 dilution, or 8 dils
R	R	R	R	N	Reactive, 1:16 dilution, or 16 dils

R = Reactive. N = Nonreactive

[1]Reactive finding with undiluted spinal fluid in the qualitative test.

4. VDRL Slide Tests with Spinal Fluid

The VDRL Spinal Fluid Slide Test was designed to be used as a substitute for the VDRL Spinal Fluid Tube Test, when the testing of a lesser volume of spinal fluid is desirable. For the VDRL Spinal Fluid Slide Test, the same 5:1 ratio of spinal fluid to emulsion of sensitized antigen is used, as in the test performed in a tube. Inasmuch as the VDRL Slide Test on serums is based on the use of an .05 ml. volume as the test dose, this same volume was selected for spinal fluid, which would require only .01 ml. of the emulsion of sensitized antigen. One-twentieth of the amount of spinal fluid and emulsion of sensitized antigen required for the VDRL spinal fluid test is used for the test. The .01 ml. of the emulsion of sensitized antigen is delivered with a needle and syringe that is calibrated to deliver 100 drops from a 1 ml. volume.

a. Equipment:

Equipment and glassware for this test are the same as listed for the VDRL Tube Tests, with the exception of the glass slide. An agglutination slide, 2 1/4 x 3 in., that contains 12 concavities (each concavity measuring 16 mm., in diameter, and 1.75 mm. in depth) is used for testing the spinal fluid.

b. Procedure:

(1) Centrifuge and decant each specimen of spinal fluid, which is tested without preliminary heating.

(2) Prepare an emulsion of sensitized antigen as described on p. 24.

(3) Pipet .05 ml. of each specimen of spinal fluid into a designated concavity of the slide.

(4) Pipet .05 ml. of appropriately diluted control serum - to yield reactive (R) diluted control serum - to yield reactive (R) and non-reactive (N) results - into two concavities.

(5) Using 23-gauge needle, with bevel, add one drop (.01 ml.) of emulsion of sensitized antigen to each specimen of spinal fluid and control.

(6) Place the slide on a rotating machine for 8 minutes, at 180 rpm. (When tests are performed in a hot, dry climate, slides may be covered during rotation with a box-lid that contains a moistened blotter, in order to prevent evaporation.)

c. Record Results:

Record results as:

REACTIVE	Definite clumping or flocculation of the particles of antigen.
NON-REACTIVE	Complete dispersion of the particles of antigen, i.e., no clumping or flocculation.

5. References:

Harris, A.; Rosenberg, A.A.; Riedel, L.M.: A microflocculation test for syphilis using cardiolipin antigen. Preliminary report. J. Ven. Dis. Inform., 27:169 -174, July 1946.

Harris, A.; Rosenberg, A.A.; Del Vecchio, E.R.: The VDRL Slide flocculation test for syphilis. II. A supplementary report. J. Ven. Dis. Inform., 29:72-75, March 1948.

Harris, A.; Rosenberg, A.A.; Del Vecchio, E.R.: A macroflocculation test for syphilis using cardiolipin-lecithin antigen. J. Ven. Dis. Inform., 29:313-316, October 1948.

Rosenberg, A.A.; Harris, A.; Harding, V.L.: A macroflocculation spinal test employing cardiolipin-lecithin antigen. J. Ven. Dis. Inform., 29:359-361, December 1948.

E. KOLMER COMPLEMENT FIXATION TESTS

1. Principle of all Complement Fixation Tests

Various modifications of the Wassermann reaction have been published. All complement fixation tests depend upon the same principle (fixation of the complement). The only difference is in the antigen; the antigen must be specific for the amboceptor-like substance present in the serum.

a. Normal rabbit serum contains a complement (heat labile) and amboceptor (heat stable).

(1) Normal rabbit serum plus sheep cells equals no hemolysis. (Heated or unheated).
(2) Sensitized rabbit serum plus sheep cells equals hemolysis. (Amboceptor plus complement).
(Rabbits sensitized by repeated injections with sheep cells).
(3) Heated sensitized rabbit serum plus sheep cells equals no hemolysis. (Amboceptor, no complement).
(Rabbit serum heated at 56°C. for 30 minutes inactivates the complement).
(4) Heated, sensitized rabbit serum plus guinea pig serum plus sheep cells
(amboceptor) (complement)
equals hemolysis.
(5) Male guinea pig serum is a most satisfactory source of complement.

b. Application of the above principles to the complement fixation procedure:

Since this antigen-antibody-complement reaction is visible, it is sometimes referred to as the "INDICATOR SYSTEM." When, as in the Kolmer test, the combination of antigen with reagin and complement is not visible the Indicator System is of use as outlined below.

Antigen plus normal serum plus guinea pig serum plus sensitized rabbit serum
(heated) (unheated) (heated)

plus sheep cells equals hemolysis.

Non-luetic serum does not contain any amboceptor-like substance that will combine with the antigen, therefore the complement is not used in the first part of the reaction and is free to be used in the second part of the reaction, where it attaches the specific amboceptor in the rabbit serum to the sheep cells and produces hemolysis of the cells.

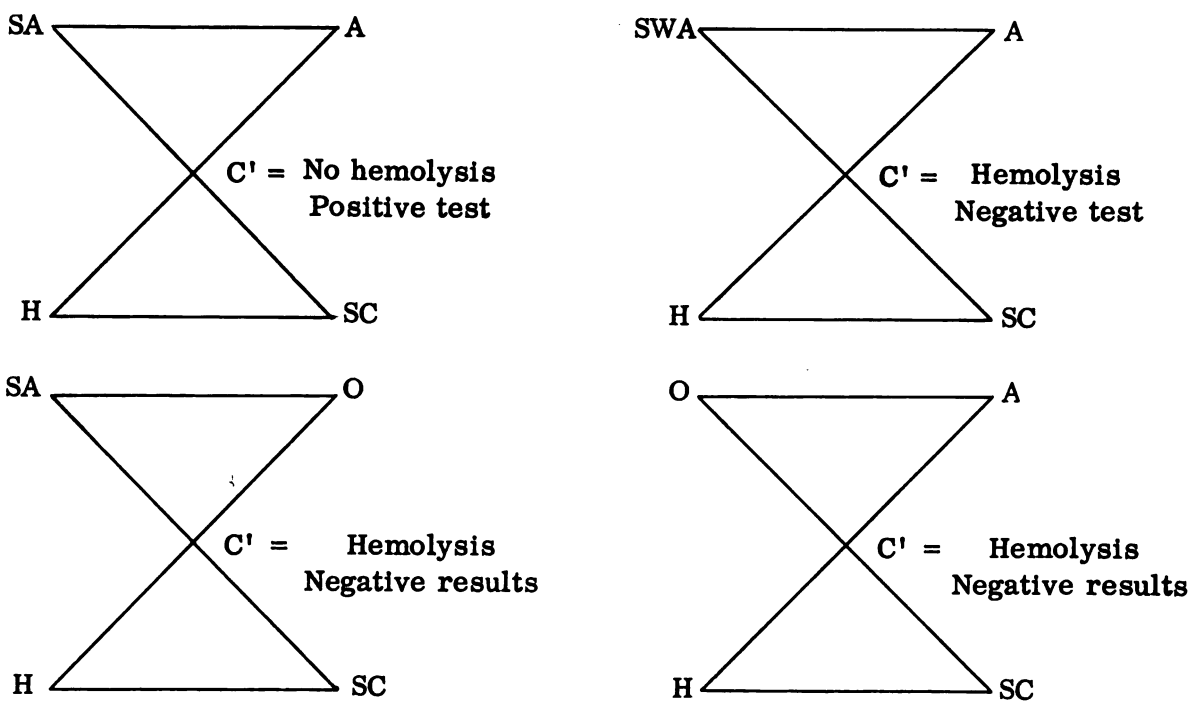
Antigen plus luetic serum plus complement plus sensitized rabbit serum plus sheep cells equals no hemolysis.

Luetic serum contains an amboceptor-like substance (in the serum globulin fraction called reagin) which is specific for the antigen, therefore the amboceptor combines with the antigen, using all the available complement from the guinea pig serum. When the sensitized rabbit serum (the hemolysin) is added plus the sheep cells, there is no available complement, therefore the amboceptor cannot produce hemolysis of the sheep cells.

The complement is used by the luetic serum and fixed to the antigen, leaving no complement available for hemolysis of the sheep cells. This is fixation of the complement.

c. In the complement fixation test the serum to be tested is heated (inactivated) to inhibit its complement. The complement in the rabbit serum (hemolysin) has been destroyed. Therefore the only source of complement is the guinea pig serum which must be carefully titrated so that a strongly positive luetic serum will utilize all of the complement. A weak luetic serum will use only part of the complement, leaving some available to produce partial hemolysis of the sheep cells.

INTERPRETATIONS: Fig. 2.



NOTE:

SA = Serum with antibody
SWA = Serum without antibody
O = Reagent missing from tube
H = Hemolysis
C' = Complement
SC = Sheep cells (2% suspension)

2. Stock Reagents

a. Hemolysin: Anti-sheep hemolysin can be prepared by giving a rabbit five to six injections of 5 ml. of a 10% suspension of washed sheep cells every three days. Bleed the rabbit 7 to 9 days after the last injection if a preliminary titration is satisfactory. Separate the serum (it need not be inactivated). Hemolysin is preserved by adding an equal volume of neutral glycerin to the rabbit serum. Employ the highest grade of glycerin. This glycerinated stock can be stored for long periods of time in the refrigerator with little loss of activity.

b. Antigen: Kolmer Antigen is composed of cardiolipin, lecithin, and cholesterol dissolved in alcohol. This antigen should be tightly stoppered and stored at room temperature in the dark. The titer of the antigen will be given on the label of the bottle.

c. Saline solution:

(1) Weigh 8.5 Gm. of dried sodium chloride (chemically pure reagent grade) and 0.1 Gm. of magnesium sulfate for each liter of saline solution. When unusually low complement titers are obtained, Kolmer recommends that 40 mg. of $CaCl_2 \cdot 2H_2O$ be added to each liter of saline solution. Under these circumstances, the complement should not be used at a dilution higher than 1:37 (for 2 full units).

(2) Dissolve salts in distilled water, and filter into flasks having glass or gauze-covered cotton stoppers. Freshly prepared saline solution should be used for each test run.

(3) Place portion of saline solution sufficient for diluting complement, to be used for completing the tests, into refrigerator, allowing remainder to stand at room temperature (73° to 85° F.).

d. Sheep Red Cells:

(1) The method of choice is to collect blood from an external jugular vein as aseptically as conditions permit. Otherwise fresh sheep blood should be collected at an abattoir.

(a) Collection in an anticoagulant and preservative solution is recommended. For this purpose 1 part of the following solution (Boerner-Lukens) for each 9 parts of blood is satisfactory:

Sodium citrate	8.0 Gm.
Dextrose	20.0 Gm.
Aqueous solution of merthiolate, 1:1,000 ...	100.0 ml.

(2) The following method (Kolmer) is likewise satisfactory:

In a clean (but not necessarily sterile) quart-sized Mason jar, place 20 ml. of 10% solution of sodium citrate in saline solution and 2 ml. of

formalin. At the abattoir have the jar filled with fresh blood, screw on top, and mix thoroughly. A bottle containing ACD solution, used for drawing blood from donors, is also satisfactory.

(3) Another satisfactory method is to dissolve 3.8 Gm. of sodium citrate (A.C.S.) in 100 ml. of distilled water. Sixty ml. of this solution is required for each 50 ml. of sheep blood collected. Sterile technic is employed in collecting and dispensing the cells which are stored at refrigerator temperature, in sterile vaccine bottles equipped with sterile rubber stoppers. The cells may remain satisfactory for use over a 6-week period.

(4) Preservation of Sheep's Blood in Alsever's Solution:

(a) Preparation of modified Alsever's solution:

Dextrose	2.0 Gm.
Sodium citrate	0.80 Gm.
Sodium chloride	0.42 Gm.
Citric acid	55 mgm.
Q.S. distilled water	100 ml.

NOTE: To obtain the quantity of each reagent, multiply by the number of mls. of distilled water to be used to get the final volume.

(b) Preparation of collection bottle:

Fill a rubber-stoppered glass bottle to the 150 ml. mark with solution, evacuate by suction, and sterilize for 15 minutes in the autoclave at 15 pounds pressure. The sterilized solution should have a final pH of about 6.1, and show little if any evidence of caramelization. Invert the bottle and mark it with wax pencil at a point which will indicate the further addition of 150 ml. of blood (final volume, 300 ml.). Fit a 20-inch length of rubber tubing with a screw clamp and two hypodermic needles, and sterilize for use as a bleeding outfit.

(c) Collection of sheep cells:

Select a site over the sheep's jugular vein; shear and paint with a 1:1000 aqueous solution of Wescodyne. Tighten the screw clamp on the rubber tubing of the bleeding outfit and insert one needle through the bottle, the other into the animal's vein. Invert the bottle so that blood will enter through the solution, and loosen the screw clamp to permit a ready flow of blood. Rotate the bottle continuously during the bleeding operation to assure thorough mixture of the components. When the bottle fills to the wax pencil mark (final vol. 300 ml.), tighten the screw clamp and withdraw the needles from bottle and vein. Discard any blood remaining in the tubing. Continue rotating the bottle for several minutes thereafter. For convenience in handling, the preserved blood may be redispensed in sterile 50 ml. rubber-stoppered bottles. Store at 3°C. to 6°C., and do not use for complement-fixation tests until at least five days after collection.

(5) Sheep cells collected by any of these methods should be kept in the refrigerator for 48 hours before they are used in a test.

e. Complement: Is known more by what it does, than by what it actually is. Found in the blood of various species of animals, the complenent in the serum of the guinea pig is most widely used. Of the four known components of complement, the best proportions of each are present in the guinea pig sera. Hemolysis requires the united effort of all four components.

Complement is a very labile substance. When submitted to a temperature of 56°C. a sudden drop in activity occurs, thus a 20 to 30 minute period of heating at that temperature is employed for the patient's sera undergoing tests. As it is believed that sera on standing may regain some of their complement activity, they must be reinactivated for 10 minutes on the day they are tested. Agitation, chemicals (acids, alkalis), and dilution also decrease the potency of complement.

For routine use, the following procedures assist in overcoming the deleterious effects mentioned in the above paragraph, and assist in keeping the complement as active as possible. When the stock vial of complement is needed and has been opened, it is kept in the refrigerator in a beaker of cold water. When the specified quantity required for titration is needed, the amount is removed and the vial of opened complement is returned immediately to the refrigerator. For actual tests, the amount required, as predetermined by the calculated complement dilution, would be the amount necessary to complete the tests, and this is prepared with the cold Kolmer saline, as was mentioned under saline solution.

Guinea pig blood may possess complement activity less than the prescribed minimum or greater than the prescribed maximum. Low complement titers may be caused by improper feeding or housing of guinea pigs, or most commonly, through loss of reactivity during storage of guinea pig serum. Complement serum stored as liquid (with preservative added) at refrigerator temperature or in the frozen state should be adequately protected from partial drying as a result of evaporation. Aliquots sufficient for 1 day's use should be placed in closed containers to avoid complement destruction due to repeated thawing and refreezing.

Some technicians are deceived by restoring dehydrated complement serum to only one-half or two-thirds of the original serum volume and then omitting the serum concentration factor when calculating complement dilution. Substandard serum may be made to appear adequately reactive in this way. This practice, used to circumvent technique restrictions, is to be discouraged.

(1) Preparation of complement:
As shown by Giordano and Carlson (Am. J. Clin. Path. 9:130, 1939),

it is advisable to pretest serums of guinea pigs individually for nonspecific reactions before use as complement in conducting complement fixation tests for syphilis. These preliminary tests should be conducted with the same cardiolipin antigen as employed in the test for syphilis because, as shown by Harris (J. Lab. & Clin. Med. 27:97, 1941), nonspecific reactions may occur with one antigen and not with another. Mixtures of serums of 100 or more guinea pigs, however, are rarely unsatisfactory. When such are used, the complement may be pretested after pooling. If found to yield nonspecific reactions with one antigen, it should be tested with others as it is extremely unlikely that any pools will be found that are not satisfactory with at least one of them. The technique employed for pretesting the complement for the Boerner-Lukens and Kolmer complement fixation tests is described below.

(a) The pooled serums of at least three to five healthy young male guinea pigs should be used. Select large, well-nourished animals that have not been fed for at least 12 hours.

(b) Cardiolipin-lecithin antigens are free of the fraction responsible for the non-specific fixation of complement at low temperature. Pretesting of guinea pig serums is therefore omitted when this type of antigen is employed.

(2) Preservation of complement:

Method 1. The most satisfactory method is to evaporate 5 ml. amounts in a vacuum by lypholization. This serum usually retains both hemolytic activity and fixability for 8 to 12 months if kept under constant refrigeration. By adding 5 ml. of aqueous solution of 6% sodium acetate, 2% boric acid, .25% benzoic acid (chemically pure grade reagent), the material is ready for use in the same manner as fresh serum.

Method 2. Add 1.0 Gm. of sodium chloride for every 10 ml. of guinea pig serum. Store in refrigerator.

Method 3. Freeze complement serum and retain in the frozen state until used.

3. Preparation for the Kolmer Tests

a. Equipment:

Galvanized wire test tube racks (72 tube capacity)
Waterbath (56°C.)
Waterbath (37°C.)
Refrigerator (6.0°C.-10°C.)
Centrifuge

b. Glassware:

Test tubes, Pyrex, 15 mm. x 85 mm.
Test tubes, centrifuge, graduated, 15 ml. capacity, Pyrex.
Tubes, centrifuge, round bottom, 50 ml. capacity.
Pipets, serological, calibrated to tip, in following sizes:
1 ml. calibrated in 1/100 ml.
5 ml. calibrated in 1/10 ml.
10 ml. calibrated in 1/10 ml.

c. Reagents:

(1) Antigen:

There is one antigen available for luetic study. It is an alcoholic solution containing cardiolipin, lecithin and cholesterol. In each case a new lot of antigen should be tested in parallel with a standard antigen, in both qualitative and quantitative tests with reactive, weakly reactive, and non-reactive sera before being placed in routine use.

(2) Refer to Stock Reagents for:
Saline Solugion (p. 37)
Sheep Cells (p. 37)
Hemolysin (p. 37)

(3) Complement:

Dehydrated complement should be restored to original serum volume by dissolving in the proper amount of buffered diluent and then stored in the refrigerator. Complement stored in the frozen state should be returned to the liquid state by incubating at room temperature or at 37°C. only long enough to melt. Since the proteins in this serum will tend to precipitate during thawing, the tubes should be adequately mixed by inversion and then returned to refrigeration.

d. Preparation of Serum:

(1) Specimens are properly labeled.

(2) The specimens are centrifuged and the serums void of cells are placed into properly labeled test tubes.

(3) The serum is inactivated by placing the tubes in a water bath at 56°C. for 30 minutes. If serum has been heated more than two hours before the actual test, it will be reheated for 10 minutes at 56°C. before proceeding with the test.

(4) Recentrifuge any serum in which visible particles have formed during heating.

NOTE: If complement fixation reactions of maximum sensitivity are desired in the Kolmer quantitative test, it is necessary to remove the natural anti-sheep hemolysins from the serum. This may be accomplished in the following manner.

(a) Pipet 1 ml. of each serum into a small (12 x 75 mm.) Kahn tube and place in the refrigerator for 15 minutes, or more.

(b) Add one drop of washed, packed, sheep red cells to each specimen and mix well.

(c) Return all tubes to the refrigerator for 15 minutes.

(d) All tubes are then centrifuged and the serum void of cells is separated by decanting.

(e) These sera are then heated at 56°C. for 30 minutes. Previously heated, absorbed sera should be reheated for 10 minutes.

e. Preparation of Spinal Fluid

(1) Centrifuge and decant all spinal fluids to remove cellular and particulate debris. Spinal fluids which are visibly contaminated or contain gross blood should not be tested.

(2) Heat all spinal fluids received through the mail, or stored for three or more days, at 56°C. for 15 minutes to remove the thermolabile anticomplementary substances.

(3) Fresh spinal fluids are tested without preliminary heating.

f. Preparation of Sheep Red Cell Suspension

(1) Filter an adequate quantity of preserved sheep blood through gauze into a 50 ml. round bottom centrifuge tube.

(2) Add two or three volumes of saline solution to each tube. Invert to mix.

(3) Centrifuge tubes at a force sufficient to pack the red cells in 10 minutes.

(4) Remove supernatant fluid by suction, using a Richard's pump, taking off also the upper white cell layer.

(5) Fill tube with saline solution and resuspend cells by inverting and gently shaking tube. Invert until all cells are off the bottom.

(6) Recentrifuge tube and repeat the process for a total of three washings. If supernatant fluid is not colorless on third washing, cells are too fragile and should not be used.

(7) After supernatant fluid is removed from third washing, cells are poured or washed into a 15 ml. graduated centrifuge tube and centrifuged at previously used speed for 10 minutes in order to pack cells firmly and evenly.

(8) Read the volume of packed cells in the centrifuge tube and carefully remove supernatant fluid. Try not to disturb cell layer.

(9) Prepare a 2% suspension of sheep cells by washing them into a flask with 49 volumes of saline solution. Shake flask to insure even suspension of cells.

Example: 2.1 ml. (packed cells) x 49 = 102.9 ml. (saline solution required).

(10) Pipet 15 ml. of the 2% cell suspension into a graduated centrifuge tube and centrifuge at previously used speed for 10 minutes. A 15 ml. aliquot of a properly prepared cell suspension will produce 0.3 ± 0.1 ml. of packed cells.

CAUTION: Use only centrifuge tubes which have been properly calibrated and checked for accuracy.

NOTE: When the packed cell volume is beyond the tolerable limits stated above, the cell suspension concentration should be adjusted. The quantity of saline solution which must be removed or added to the cell suspension to accomplish this is determined according to the following formula:

$$\frac{\text{Actual reading of centrifuge tube}}{\text{Correct reading of centrifuge tube}} \times \text{Volume of cell suspension} = \text{Corrected volume of cell suspension}$$

Example 1:

Volume of cell suspension ..... 100 ml.
Centrifuge tube (15 ml.) reading.... 0.27 ml.

$$\frac{0.27 \text{ ml.}}{0.3 \text{ ml.}} \times 100 \text{ ml.} = 90 \text{ ml.}$$

Therefore, 10 ml. of saline solution should be removed from each 100 ml. of cell suspension. Saline solution may be removed by centrifuging an aliquot of the cell suspension and pipetting off the desired volume of saline solution for discard.

Example 2:

Volume of cell suspension.....100 ml.
Centrifuge tube (15 ml.) reading ....0.33 ml.

$$\frac{0.33 \text{ ml.}}{0.3 \text{ ml.}} \times 100 \text{ ml.} = 110 \text{ ml.}$$

Therefore, 10 ml. of saline solution should be added to each 100 ml. of cell suspension. An adjusted cell suspension should be rechecked by centrifuging a 15 ml. portion.

(11) Place flask of cell suspension in refrigerator when not in use. Always gently shake before using to insure an even suspension, since the red cells settle to the bottom of the flask when allowed to stand.

g. Preparation of Antigen Dilution:

(1) Place the required amount of saline solution in a flask and add antigen drop by drop while continuously shaking the flask. The amount needed may be calculated from the number of tubes containing antigen in the tests and titrations. The test dose consitutues 0.5 ml. of the antigen dilution indicated on the lable of the bottle.

(2) Diluted antigen is kept in the dark at room temperature in a stoppered flask.

(3) Diluted cardiolipin antigen should stand at room temperature for at least ONE HOUR before it is used. During this interval the cardiolipin antigen will become more opalescent. The titer of cardiolipin antigen is usually 1:150 (commercial sources).

(4) Formula of Kolmer's cardiolipin antigen:

An alcoholic solution containing:

Cardiolipin - 0.03%
Lecithin - 0.05%
Cholesterol - 0.3%

h. Preparation of Stock Hemolysin Dilution

(1) Prepare 1:100 stock hemolysin dilution as follows:

Saline solution	94.0 ml.
Phenol solution (5% in saline solution)	4.0 ml.
Glycerinized hemolysin (50%)	2.0 ml.

Phenol solution should be mixed well with the saline solution before glycerinized hemolysin is added. This solution keeps well at refrigerator temperature but should be discarded when found to contain precipitate.

(2) Each new lot of stock hemolysin dilution (1:100) should be checked by parallel titration with the previous stock hemolysin dilution before it is placed into routine use.

(3) Dilutions of hemolysin of 1:1,000 or greater are prepared by further diluting aliquots of the 1:100 dilution.

After these reagents are prepared the complement and hemolysin titrations may be assembled.

i. Complement and Hemolysin Titrations

Perform these two titrations simultaneously in the same rack.

(1) Place 10 tubes appropriately numbered 1,000 to 16,000 in one side of the rack for the hemolysin titration and 8 tubes appropriately numbered as to ml. of complement, in the other side for the complement titration. Add two other tubes to the rack, one for 1:1,000 hemolysin dilution and one for 1:30 complement dilution.

NOTE: The 10 tubes for the hemolysin titration are numbered according to the dilution ratios, i.e., the first six tubes numbered 1,000 to 6,000 and the last four numbered 8,000; 10,000; 12,000; and 16,000.

Of the 8 tubes for complement titration 7 are numbered in ml. of complement , i.e., 0.2, 0.25, 0.3, etc., up to 0.5 ml, the 8th tube being identified by 0.0.

(2) Prepare a 1:1,000 dilution of hemolysin by placing 4.5 ml. of saline solution in a test tube and adding 0.5 ml. of 1:100 stock hemolysin solution. Mix well.

(a) Pipet 0.5 ml. of 1:1,000 hemolysin dilution into the first five tubes of the hemolysin titration.

(b) Add the following amounts of saline solution to the hemolysin titration tubes:

Table 5.

Tube Identification	(read in thousands, i.e., 1,000 etc.) 1	2	3	4	5	6	8	10	12	16
Saline solution	None	0.5 ml.	1.0 ml.	1.5 ml.	2.0 ml.	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.

For speed and accuracy in the addition of saline a 5.0 ml. (graduated serological) pipet should be used. The readings of the pipet upon successive deliveries of saline starting with tube 2 through 4 are: 0.5, 1.5, 3.0. Refill the pipet, add 2.0 ml. to tube 5 and 0.5 ml. to the rest of the tubes. In serological technic, it is not good practice to use the last 0.5 ml. of fluid at the tip of the pipet.

(c) Proceed as follows:

Table 6.

Tube	Process		Final hemolysin dilution
1st (1,000)	Mix. None		1:1,000
2nd (2,000)	Mix. Discard 0.5 ml.		1:2,000
3rd (3,000)	Mix. Transfer 0.5 ml. to tube 6	Discard 0.5 ml.	1:3,000
4th (4,000)	Mix. Transfer 0.5 ml. to tube 8	Discard 1.0 ml.	1:4,000
5th (5,000)	Mix. Transfer 0.5 ml. to tube 10	Discard 1.5 ml.	1:5,000
6th (6,000)	Mix. Transfer 0.5 ml. to tube 12		1:6,000
7th (8,000)	Mix. Transfer 0.5 ml. to tube 16		1:8,000
8th (10,000)	Mix. Discard 0.5 ml.		1:10,000
9th (12,000)	Mix. Discard 0.5 ml.		1:12,000
10th (16,000)	Mix. Discard 0.5 ml.		1:16,000

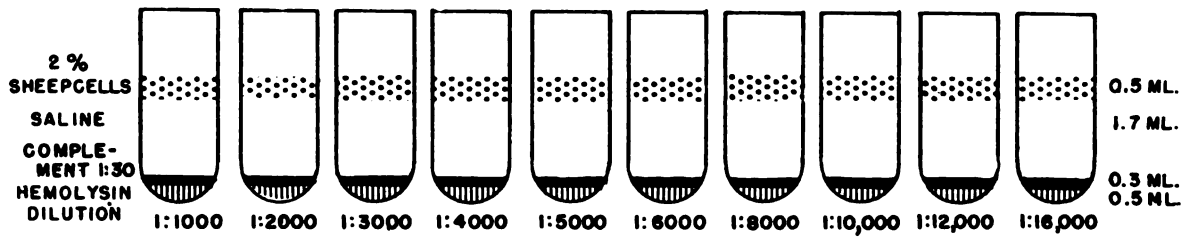
To assure a good mixing of the serum and saline, it is good technic to draw up the serum-saline mixture into the pipet and expel it back into the tube at least three times.

In making the transfers beginning with tube 3 to tube 6, tube 4 to 8, etc., two tubes are skipped each time. By placing a finger over the mouths of the tubes to be skipped, the chance of error due to incorrect dilution is reduced.

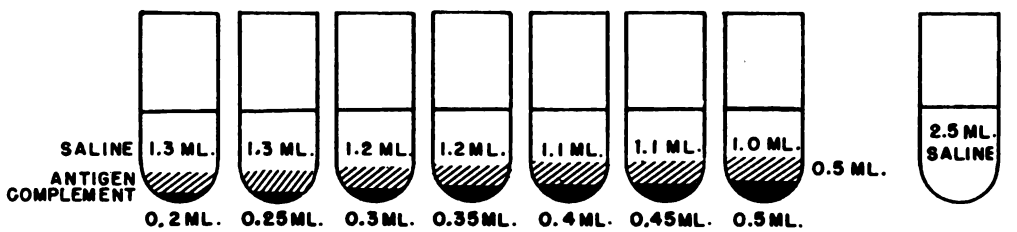
Table 7.

SCHEMATIC REPRESENTATION

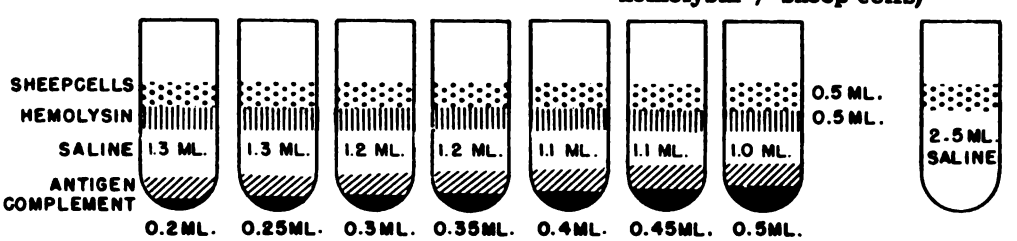
I. HEMOLYSIN TITRATION



II. A. COMPLEMENT TITRATION - First Stage

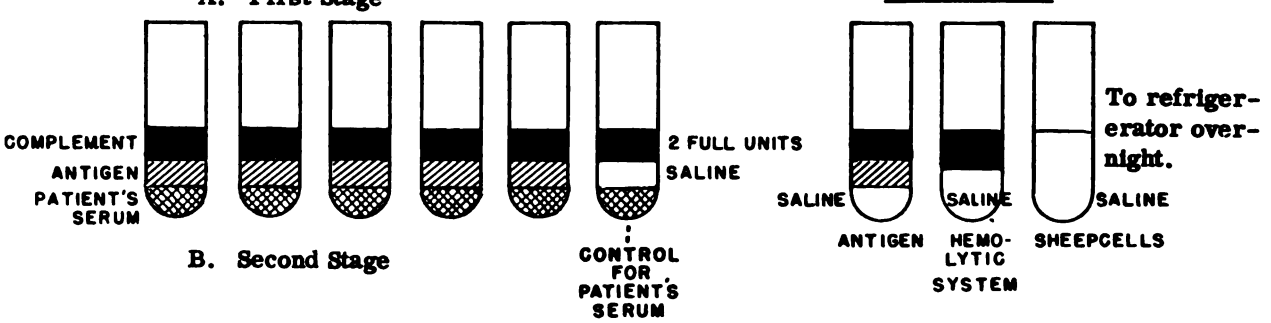


B. COMPLEMENT TITRATION - Second Stage (incubation with hemolysin + sheep cells)



III. KOLMER COMPLEMENT FIXATION TEST

A. First Stage



B. Second Stage

ADD HEMOLYSIN (2 UNITS) TO ALL TUBES EXCEPT SHEEP CELL CONTROL. ADD SHEEP CELLS TO ALL TUBES.

HEMOLYSIN ANTIGEN

COMPLEMENT SHEEP CELLS SERUM OF PT. SALINE

(3) Prepare 1:30 dilution of complement by adding 0.2 ml. of guinea pig serum to 5.8 ml. of saline solution and mixing well.

(a) Use a 5 ml. pipet. Draw saline to the 0.0 mark and release 4 ml. into tube. Refill pipet and add 1.8 ml. to the tube thereby avoiding using the last 0.5 ml. at the tip of the pipet.

(4) Pipet 0.3 ml. of 1:30 complement into each of 10 tubes of the hemolysin titration.

(5) Add the following amounts of 1:30 complement to the bottom complement titration tubes.

Table 8.

COMPLEMENT 1:30 - 8 tubes								
Tube Identification	0.2 ml.	0.25 ml.	0.3 ml.	0.35 ml.	0.4 ml.	0.45 ml.	0.5 ml.	0.0 ml.

Successive readings of the 1.0 ml. (graduated serological) delivery pipet will be 0.2, 0.45, 0.75. Refill the pipet for delivery readings of 0.35, 0.75. Refill the pipet for delivery readings of 0.45 and 0.95.

(6) Add 0.5 ml. of antigen dilution to each of the first seven tubes of the complement titration.

(7) Add 1.7 ml. of saline solution to each of the 10 tubes of the hemolysin titration. For speed in the addition of 1.7 ml. of saline to each tube, a 10 ml. (graduated serological) pipet is used. This is the only time in the Kolmer test where the use of a 10 ml. pipet is advised. Readings upon delivery of 1.7 ml. of saline to successive tubes will be 1.7, 3.4, 5.1, 6.8, 8.5. Refill the pipet and repeat.

(8) Add the following amounts of saline solution to the complement titration tubes.

Table 9.

Tube Identification (ml. of complement)	0.2	0.25	0.3	0.35	0.4	0.45	0.5	0.0
Saline Solution	1.3 ml.	1.3 ml.	1.2 ml.	1.2 ml.	1.1 ml.	1.1 ml.	1.0 ml.	2.5 ml.

For addition of saline a 5.0 ml. (graduated serological) pipet is employed. The readings of the pipet on progressive delivery of saline to the respective tubes will be 1.3, 2.6, 3.8. Refill the pipet and deliver 1.2 to tube 0.35, next reading for tube 0.4 and successive deliveries will be 2.3, 3.4, 4.4. Refill the pipet to deliver 2.5 ml. to tube 0.0 (the 8th tube).

(9) At this point, the complement titration and the completed hemolysin titration stand as shown in Tables 10 and 11.

Table 10. Complement titration (first stage)

Tubes (identified in ml. of complement)	Complement	Antigen	Saline
1st 0.2	0.2	0.5	1.3
2nd 0.25	0.25	0.5	1.3
3rd 0.3	0.3	0.5	1.2
4th 0.35	0.35	0.5	1.2
5th 0.4	0.4	0.5	1.1
6th 0.45	0.45	0.5	1.1
7th 0.5	0.5	0.5	1.0
8th 0.0	0.0	0.0	2.5

Table 11. Hemolysin titration (complete)

Tubes (identification)		Hemolysin	Complement	Saline	Sheep Cell suspension -2%
1st	1,000	1:1,000	0.3	1.7	0.5
2nd	2,000	1:2,000	0.3	1.7	0.5
3rd	3,000	1:3,000	0.3	1.7	0.5
4th	4,000	1:4,000	0.3	1.7	0.5
5th	5,000	1:5,000	0.3	1.7	0.5
6th	6,000	1:6,000	0.3	1.7	0.5
7th	8,000	1:8,000	0.3	1.7	0.5
8th	10,000	1:10,000	0.3	1.7	0.5
9th	12,000	1:12,000	0.3	1.7	0.5
10th	1:16,000	1:16,000	0.3	1.7	0.5

(10) Add 0.5 ml. of 2% sheep red cell suspension to each tube of the hemolysin titration.

(a) Shake each tube of the hemolysin titration to insure even distribution of cells and place rack containing the two titrations in the 37°C. water bath for 1 hour.

(11) Remove rack from water bath and read hemolysin titration.

The unit of hemolysin is the highest dilution that gives complete sparkling hemolysis.

NOTE: This means when all sheep cells are hemolyzed. If endpoint of clear, sparkling hemolysis is questionable, place tube in centrifuge. Spin at 2,000 rpm for 10 minutes. If cells are present in tube, this is not the end point of titration.

(a) Hemolysin for the complement titration and test proper is diluted so that 2 units are contained in 0.5 ml.

(12) Prepare a quantity of diluted hemolysin, containing 2 units per 0.5 ml. sufficient for the complement titration and the tests proper plus the controls in accordance with Table 12. Place the diluted hemolysin in the refrigerator when not in use.

Table 12. Preparation of Hemolysin Dilution

Dilution containing 1 unit per 0.5 ml.	Dilution containing 2 units per 0.5 ml.	To prepare 2 unit hemolysin dilution	
		1:100 Hemolysin	Saline solution
1:4,000	1:2,000	0.3	5.7
1:5,000	1:2,500	0.2	4.8
1:6,000	1:3,000	0.2	5.8
1:8,000	1:4,000	0.15	5.85
1:10,000	1:5,000	0.1	4.9
1:12,000	1:6,000	0.1	5.9
1:16,000	1:8,000	0.1	7.9

(13) Add 0.5 ml. of diluted hemolysin (containing 2 units of hemolysin) to each of the first seven tubes of the complement titration.

(14) Add 0.5 ml. of 2% sheep red cell suspension to all eight tubes of the complement titration. The addition of hemolysin and cells to the complement titration should be completed without delay, preferably within 5 minutes after rack is removed from the water bath.

(15) Shake each tube of the complement titration to insure even distribution of cells and return to the 37°C. water bath for 30 minutes.

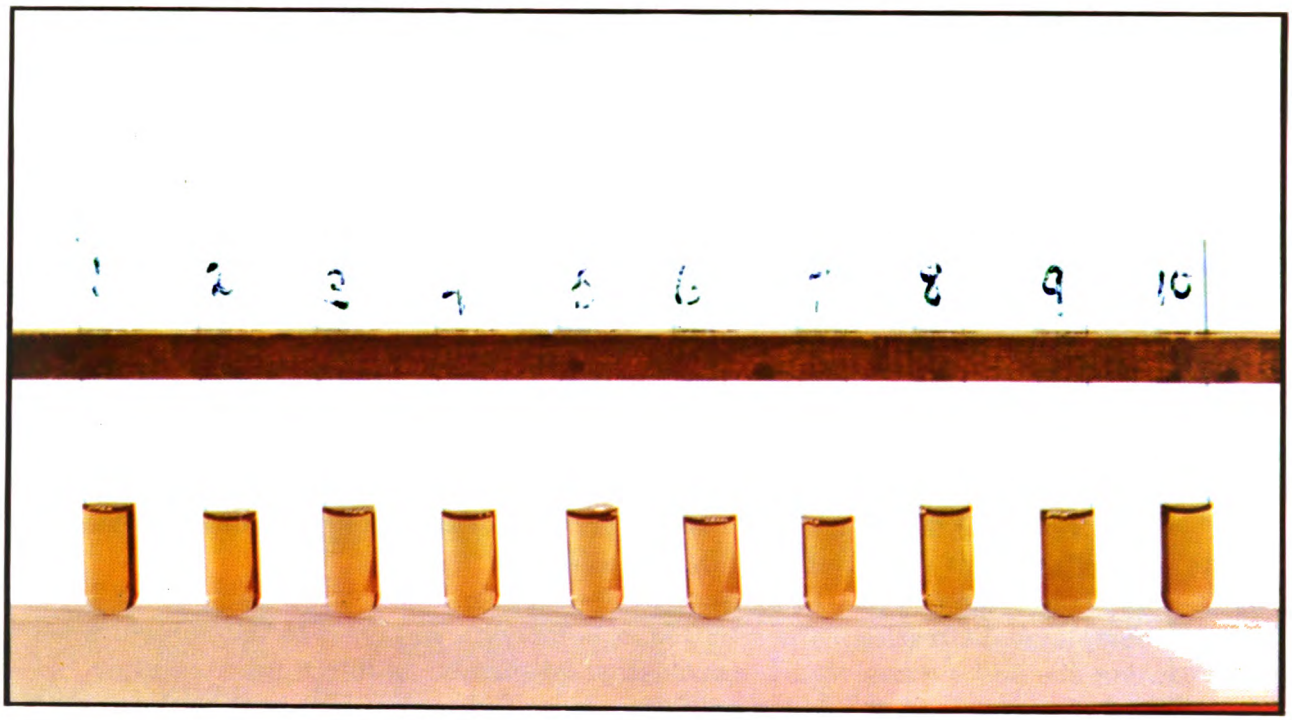
The completed complement titration is shown below.

Table 13. Complement titration (complete)

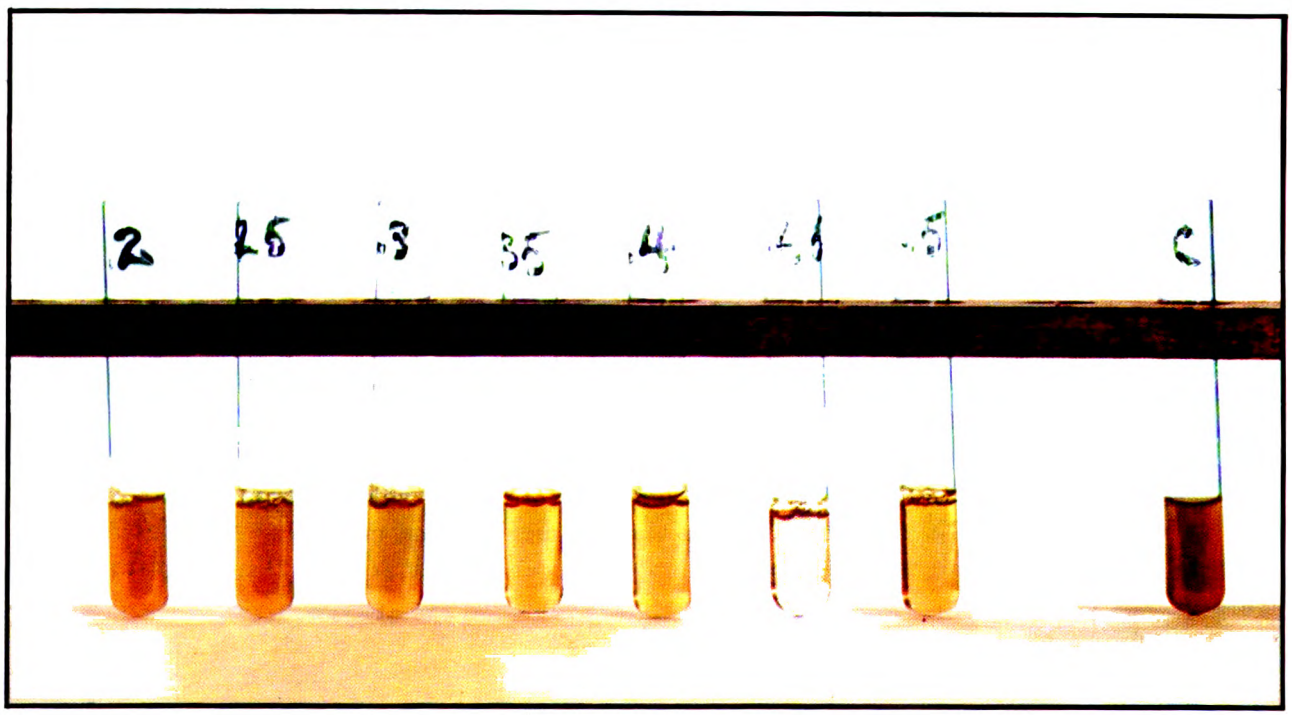
Tubes (identified in ml. compl.)		Complement 1:30	Antigen solution	Saline solution		Hemolysin	Sheep cell suspension (2%)	
1st	0.2	0.20	0.5	1.3	37°C. water bath for 30 min.	0.5	0.5	37°C. water bath for 30 min.
2nd	0.25	0.25	0.5	1.3		0.5	0.5	
3rd	0.3	0.30	0.5	1.2		0.5	0.5	
4th	0.35	0.35	0.5	1.2		0.5	0.5	
5th	0.4	0.40	0.5	1.1		0.5	0.5	
6th	0.45	0.45	0.5	1.1		0.5	0.5	
7th	0.5	0.50	0.5	1.0		0.5	0.5	
8th	0.0	None	None	2.5		None	0.5	

(16) Remove rack from water bath and read complement titration.

The smallest amount of complement giving complete sparkling hemolysis is the exact unit. The full unit is 0.05 ml. more than the exact unit.



Hemolysin Titration



Complement Titration

(17) For the complement fixation tests, complement is diluted so that 2 full units are contained in 1.0 ml.

Example:	ml.
Exact unit	0.3
Full unit	0.35
Dose (2 full units)	0.7

Dilution of complement to be employed in the test proper may be calculated by dividing 30 by the dose, i.e., $\frac{30}{0.7}$ = 43 or 1:43 dilution of complement (guinea pig serum).

Table 14 gives additional examples.

Table 14. Preparation of Complement Dilution

Exact Unit	Full unit	Dose	Dilution to use	Preparation		
				Guinea pig serum		Saline Solution
0.3	0.35	0.7	1:43	1	+	42
0.35	0.4	0.8	1:37	1	+	36
0.4	0.45	0.9	1:33	1	+	32
0.45	0.50	1.0	1:30	1	+	29

Occasionally hyperactive guinea pig serums are encountered which yield titrations indicating 2 full units per milliliter in dilutions greater than 1:43. These guinea pig serums should be used at 1:43 dilution to accomplish satisfactory testing.

NOTE: Tubes of the complement or hemolysin titrations showing complete hemolysis will be removed and placed in the refrigerator for use as hemoglobin solutions in preparing the reading standards.

4. Kolmer Qualitative Tests with Serum and Spinal Fluid

Arrange test tubes in wire racks so that there are three tubes for each serum and spinal fluid to be tested. Control serums of graded reactivity should be included. Number the first row of tubes to correspond to the serum and spinal fluid being tested. Three additional test tubes are included for reagent controls.

a. Pipet 0.45 ml. of Kolmer saline into each tube of the first row for serum. No saline is used in the first tube of spinal fluid. Pipet 0.25 ml. into each tube of the second and third rows for both serum and spinal fluid.

b. Add the following amounts of saline solution to the three reagent control tubes:

Antigen control (for serum tests)	0.25 ml.
Hemolytic system control	0.50 ml.
Red cell control	1.25 ml.

NOTE: Unless the Qualitative Test is run separately, the control system is not set.

c. Pipet:
0.3 ml. of each serum to be tested into tube 1;
0.25 ml. of each spinal fluid to be tested into tubes 1, 2 and 3; discard 0.25 ml. from tube 2.
0.25 ml. of the 1:1 dilution into tubes 2 and 3.

d. Add 0.25 ml. of the antigen dilution to tubes 1 and 2 of each test, either serum, control serum, or spinal fluid, and the antigen control tube.

e. Allow test racks to stand for not less than 10 minutes, nor more than 30 minutes, at room temperature before addition of diluted complement.

f. Prepare diluted complement during this interval. The amount needed is equivalent to 0.5 ml. for each tube of the test plus a slight excess.

NOTE: The volume of guinea pig serum to be diluted is determined by the amount of diluted complement necessary for the test proper. Dividing the number of milliliters of diluted complement needed by the titration dilution factor (two full units) will give the number of milliliters of guinea pig serum needed. Calculations may be made in accordance with Table below.

Table 15. Volume Determination Method

Complement dilution required	Diluted complement required	Guinia pig serum required	Cold saline solution required
ml.	ml.	ml.	ml.
1:43	43	1	42
1:43	215	5	210
1:37	37	1	36
1:30	210	7	203

g. Add 0.5 ml. of diluted complement (containing 2 full units) to all tubes of the serum, control serum, and spinal fluid tests, including the antigen control tube and the hemolytic system control tube.

h. Mix the contents of the tubes by shaking the racks well and place in the refrigerator at 6°C. to 10°C. for 15 to 18 hours.

i. Remove racks of tubes from the refrigerator and place immediately in the 37°C. water bath for 10 minutes. The interval will be determined by the length of time necessary to add hemolysin and sheep cell suspension to each rack.

j. Remove each rack from the water bath and add 0.25 ml. of the diluted hemolysin to all tubes of the test except the sheep red cell control tube.

k. Add 0.25 ml. of the 2% sheep red cell suspension (prepared the previous day) to all tubes. The 2% cell suspension should be agitated occasionally to insure even suspension of cells during the period when this reagent is being added to the complement fixation tests.

l. Mix the contents of the tubes thoroughly by shaking each rack before returning it to the 37°C. water bath for the secondary incubation. Examine the controls at 5 minute intervals. The period of secondary incubation will be determined by the length of time necessary to reproduce the predetermined reactivity pattern of the control serums. In all instances, however, the reading time should be at least 10 minutes more than is required to hemolyze the antigen and hemolytic system controls but should not exceed a total of 60 minutes incubation.

Table 16. Outline of Kolmer Qualitative Tests with Serum and Spinal Fluid

Tube No.	Kolmer Saline (ml.)	Serum or Spinal Fluid		Antigen (ml.)		Complement Two full units (ml.)		Hemolysin Two full units (ml.)	Sheep Red cell 2% (ml.)	
Serum		Serum (ml.)								
1	0.45	0.30	Mix and transfer 0.25ml. from tube 1 to tubes 2 and 3. Mix and discard 0.25ml. from tube 2.	0.25	Shake rack well. Allow to stand at room temperature for 10 to 30 minutes.	0.50	Shake rack well. Primary incubation 15 to 18 hours at 6°C. to 10°C. Remove and incubate in 37°C. water bath for 10 minutes.	0.25	0.25	Shake rack well. Secondary incubation in 37°C. water bath.
2	0.25			0.25		0.50		0.25	0.25	
3	0.25					0.50		0.25	0.25	
Spinal Fluid		Spinal Fluid (ml.)								
1	None	0.25	Mix and discard 0.25ml. from tube 2	0.25		0.50		0.25	0.25	
2	0.25	0.25		0.25		0.50		0.25	0.25	
3	0.25	0.25				0.50		0.25	0.25	

m. Remove each rack of tubes from the water bath at the end of the secondary incubation period. Record observed hemolysis as described under "Preparation of Reading Standards" and "Reading and Reporting Test Results" except in those instances where inhibition of hemolysis is noted in the control tube.

n. All serums and spinal fluids showing inhibition of hemolysis in the control tube should be returned to the 37°C. water bath for a period sufficient to complete 1 hour of secondary incubation. At the end of this period, these tests are removed from the water bath, and the tube readings (including control tubes) are recorded.

5. Kolmer Quantitative Tests with Serum and Spinal Fluid using Cardiolipin Antigen

a. Place test tubes in wire racks, allowing eight tubes for each serum and spinal fluid to be tested; arrange so that the front row contains tubes 1, the second row tubes 2, etc., of each specimen, including tubes for positive and negative controls. Allow one tube each for the antigen, hemolysin, and sheep cell controls.

b. Pipet the following amounts of Kolmer saline into eight tubes, for each serum, starting with tube 1 in the front row: 0.45 ml., 0.25 ml., 0.25 ml., 0.25 ml., 0.25 ml., 0.25 ml., 0.25 ml., and 0.25 ml.

c. Pipet the indicated amounts of saline into each of the following reagents control tubes:

Antigen control	0.25 ml.	saline
Hemolysin control	0.50 ml.	"
Sheep cell control	1.25 ml.	"

d. For each serum which has been inactivated and had the anti-sheep agglutinin removed, add 0.3 ml. to tube 1. Mix and transfer 0.25 ml. to tube 2, and 0.25 ml. to tube 8 (which is the control tube).

Mix tube 2 and transfer 0.25 ml. to tube 3.
Mix tube 3 and transfer 0.25 ml. to tube 4.
Mix tube 4 and transfer 0.25 ml. to tube 5.
Mix tube 5 and transfer 0.25 ml. to tube 6.
Mix tube 6 and transfer 0.25 ml. to tube 7.
Mix tube 7 and discard 0.25 ml. from tube 7.

Table 17. Outline of Kolmer Quantitative Test with Serum or Spinal Fluid

Tube No.	Kolmer Saline	Serum or Spinal Fluid		Final Dilution	Antigen		Complement Two Full Units		Hemolysin - 2 Full Units	Sheep Red Cell Suspension 2%
	(ml.)	(ml.)			(ml.)		(ml.)		(ml.)	(ml.)
1	0.45	0.3	Mix and transfer 0.25 ml. to tube 2 and 8 from 1. Mix and transfer consecutively 0.25 ml. from tube 2 to tube 3, discarding 0.25 ml. from tube 7.	1:1	0.25	Shake rack well. Allow to stand at room temperature for 10 to 30 minutes.	0.50	Shake rack. Refrigerate 15 to 18 hours. Place in 37°C. water bath for 10 minutes.	0.25	0.25
2	0.25			1:2	0.25		0.50		0.25	0.25
3	0.25			1:4	0.25		0.50		0.25	0.25
4	0.25			1:8	0.25		0.50		0.25	0.25
5	0.25			1:16	0.25		0.50		0.25	0.25
6	0.25			1:32	0.25		0.50		0.25	0.25
7	0.25			1:64	0.25		0.50		0.25	0.25
8	0.25			Control	None		0.50		0.25	0.25

CONTROLS							
	Kolmer Saline	Serum or Spinal Fl.					
Antigen	0.25 ml.	None	0.25		0.50		0.25	0.25
Hemolysin	0.50 ml.	None	None		0.50		0.25	0.25
Red Cell	1.25 ml.	None	None		None		None	0.25

6. Preparation of Reading Standards

a. Heat tubes of hemoglobin solution (saved from the titration or obtained from control tubes of current day's tests) in the 56°C. water bath for 5 minutes. (See NOTE under Table 14, p. 51.)

b. Prepare a 1:6 dilution of a 2% red cell suspension by adding 5 ml. of saline solution to 1 ml. of 2% suspension.

c. Prepare reading standards by mixing hemoglobin solution and cell suspension in the proportions given in Table 18, page 56.

d. Reading standards are prepared with one-half volumes of cell suspension and hemoglobin solution when performing the one-half volume tests.

Table 18. Preparation of Reading Standards

Red Cell Suspension 1:6	Hemoglobin solution	Equivalent complement fixation	
		Per cent	Reading
(ml.)	(ml.)		
3.0	0.0	100	4+
1.5	1.5	50	3+
0.75	2.25	25	2+
0.3	2.7	10	1+
0.15	2.85	5	±
	3.0	0	-

7. Reading and Reporting Test Results

a. All serum and spinal fluid controls should show complete hemolysis.

b. Estimate the individual tube readings by comparison with the reading standards at the end of the secondary incubation period, and record degree of complement fixation noted, except for those specimens showing inhibition of hemolysis in the control tube.

c. Read the tubes that have been returned to the 37°C. water bath for a full hour's secondary incubation, estimating and recording the degree of complement fixation of each tube and control tube by comparison with the reading standards.

d. Report the results of the qualitative tests in accordance with the table below.

Table 19. Reporting Test Results

Test tube reading	Control tube reading	Report	Test tube reading	Control tube reading	Report
4+	-	Reactive (positive)	3+	3+	Anticomplementary (A)
3+	-	Reactive (positive)	3+	2+	Anticomplementary (A)
2+	-	Reactive (positive)	3+	1+	Anticomplementary (A)
1+	-	Reactive (positive)	3+	±	Anticomplementary (A)
±	-	Weakly reactive (D)	2+	2+	Anticomplementary (A)
-	-	Nonreactive (neg)	2+	1+	Anticomplementary (A)
4+	4+	Anticomplementary (A)	2+	±	Anticomplementary (A)
4+	3+	Anticomplementary (A)	1+	1+	Anticomplementary (A)
4+	2+	Anticomplementary (A)	±	±	Anticomplementary (A)
4+	1+	Anticomplementary (A)	-		

D - doubtful; A - anticomplementary; neg. - negative

e. Quantitative tests are reported in terms of the highest dilution giving a 1+, 2+, 3+ or 4+ reading. The only time that a plus-minus (±) is considered in the titer is when it appears in the first tube of the series. In this case when all higher dilutions are negative the result is reported as weakly reactive (D).

Serum or spinal fluids

Undiluted	Dilutions				
1:1	1:2	1:4	1:8	1:16 etc.	
4	4	4	4	4	Reactive 16 or more dils.
4	3	2	1	±	Reactive 8 dils
±	-	-	-	-	Weakly Reactive
1	±	-	-	-	Reactive undiluted or 1 dil.
-	-	-	-	-	Non-Reactive

Kolmer Units are computed by the formula S = 4D, where S is the serum or spinal fluid potency and D is the highest dilution giving a positive reaction.

8. Sources of Error

a. Complement:

When difficulties are experienced, they are usually first ascribed to defective hemolysin or antigen, but since both of these keep very well, they are rarely responsible. In some cases difficulties are due to the use of a complement too low in hemolytic activity and particularly in the case of a preserved complement. This is especially true during the hot months of the year. Under these conditions anticomplementary reactions may occur with incomplete hemolysis of the antigen, serum, and spinal fluid controls. When the unit of complement is more than 0.5 ml. of 1:30 dilution in the regular tests, it should not be used.

Furthermore, while the complement may be satisfactory from the standpoint of hemolytic activity, it may be defective because it is supersensitive to what may be called the occult anticomplementary effects of antigen, serum, or spinal fluid. Under these circumstances inhibition of hemolysis occurs in those tubes carrying antigen, while the antigen, serum, and spinal fluid controls show complete hemolysis with the danger of reporting falsely positive or nonspecific reactions. When the pretested complement or the pooled complement of a large number of guinea pigs is employed, the occult anticomplementary effects are exceptional occurrences. However, since they may happen in quantitative and qualitative spinal fluid tests, it is recommended that egg albumin be used routinely in conducting these tests, especially if the pretested complement is not being used.

b. Prezone Reactions:

Prezone reactions may occur in quantitative tests with serums, giving reactions like - - 2 3 4, with complete hemolysis of the serum control, but sometimes with incomplete hemolysis of the antigen control. They occur rather infrequently when the pretested or pooled complement is being used. These may be prevented by the use of 50% egg albumin. Zonal reactions may occur if the normal anti-sheep cell agglutinins have not been absorbed from the patient's serum.

c. Hemolysin:

As previously stated, this is usually first suspected but is generally least likely to be the cause of difficulty, especially if the hemolysin has been previously found to be satisfactory. The unit of antisheep hemolysin in the regular tests should be at least 0.5 ml. of 1:4,000. Hemolysins of this and higher strength are so easy to prepare that it is a mistake to use weaker products. If the saline solution and complement are satisfactory, a good hemolysin is rarely responsible, even when shipped over long distances or kept in a refrigerator over months or even years.

d. Red cells:

When blood is obtained from an abbatoir, the red cells of occasional animals possessing increased resistance to serum hemolysis are almost always found. The cause of this phenomenon is unknown; fortunately it is rare. The remedy is to discard the red cells and secure a fresh supply of blood. When the corpuscles of preserved blood tend to become too fragile, it is advisable to discard them.

e. Antigen:

Provided no mistakes have occurred in dilution and dosage, antigen is very rarely the cause of trouble. When the antigen control shows incomplete hemolysis, it is almost surely due to some component of the hemolytic system, especially a complement supersensitive to antigen, in which case egg albumin may be used.

f. Anticomplementary Serums and Spinal Fluids:

Serums and spinal fluids may be found to be anticomplementary, as shown by incomplete hemolysis of the controls. It is much safer and wiser to repeat the tests with fresh serum or spinal fluid, especially in the case of those technicians lacking experience in complement fixation work. It is infinitely better to repeat the test than to run the slightest chance of error, especially the regrettable error of rendering a falsely positive reaction. Sometimes the majority of

serums or spinal fluids of a day's work show incomplete hemolysis of the controls, but this trouble is not due to anticomplementary effects on their part but rather to the use of a defective supersensitive complement. Under these conditions the tests will be repeated and for this reason the unused portion of the serums and of spinal fluids should always be kept in the refrigerator until the test is completed, in case repetitions are required.

F. MAZZINI MICROFLOCCULATION TEST WITH SERUM

The outline below is the Mazzini Microflocculation Test employing lipoidal antigen. This has proven of value in a battery of tests, although for routine procedures the Mazzini Test employing cardiolipin antigen may be more reliable.

1. Equipment

A rotating machine, ring maker, slide holder, 30 ml. glass-stoppered round bottle, a 5 ml. syringe, and a 25-gauge needle are necessary.

2. Reagents

Mazzini cholesterolized antigen and buffered 1% saline solution, pH 6.3 to 6.4 (obtained with antigen) are used.

3. Preparation of Serums

Same as for other serologic tests.

4. Preparation of Antigen Suspension

a. Pipet 3.0 ml. of buffered saline to the bottom of a 30 ml. bottle.

b. Measure 0.4 ml. of cholesterolized antigen (with a 1 ml. pipet graduated to the tip). For only a few tests, 1.5 ml. of buffered saline may be used with 0.2 ml. of antigen.

c. Hold the bottle in the left hand and impart a rapid rotating motion to it as the antigen is being discharged directly and at once into the saline solution from the pipet held in the right hand (antigen is blown from pipet).

d. Mix by drawing the suspension into the pipet and blowing it out two or three times.

e. Allow the suspension to "ripen" at room temperature in a dark place for 3 hours. (This suspension is usuable during the working day.)

f. Shake the antigen suspension gently from the bottom to the top of the bottle several times.

g. Transfer the suspension to the 5 ml. syringe fitted with a 25 gauge needle which has been previously calibrated to deliver 0.01 ml. of antigen suspension per drop, when held in a vertical position.

5. Performance of Test

a. To a paraffin-ringed glass slide pipet 0.05 ml. of previously inactivated serum.

b. To the serum add 1 drop of previously prepared antigen suspension.

c. Rotate for 4 minutes at 120 rpm by hand (when rotating the slide by hand, circumscribe a 2 inch circle) or 180 rpm on Boerner type mechanical rotator.

d. Check for clumping under a low power microscope using 100 x magnification.

No clumping	Negative
Very small clumps	One plus
Small clumps	Two plus
Medium clumps	Three plus
Large clumps	Four plus

e. Add a second drop of antigen to each test that gives a 1+, 2+, or atypical reaction, rotate slide for 4 minutes, and make a second reading.

f. Examine microscopically. If the reading is stronger, record the result; if weaker, record the original result.

NOTE: Positive and negative controls are required.

g. Report results as follows:

Readings	Report
Negative	Nonreactive (Negative)
1+	Weakly reactive (Doubtful)
2+	Weakly reactive (Doubtful)
3+	Reactive (Positive)
4+	Reactive (Positive)

G. REITER PROTEIN COMPLEMENT FIXATION TEST

1. Introduction:

Ryprogen is a lyophilized, serologically lipid-free, highly purified protein antigen prepared from extracts of Treponema pallidum (Reiter Strain) by the method of D'Alessandro and de Bruijn at the National Institute of Public Health, Utrecht, The Netherlands. It may be used for either qualitative or quantitative tests for syphilis in sera or spinal fluids in a conventional one-fifth volume Kolmer-type complement fixation test.

2. Reagents:

Each ampule of Reiter Protein Antigen contains sufficient antigen for 800 qualitative one-fifth volume Kolmer-type complement fixation tests.

a. Reiter Protein Antigen is reconstituted by adding 1.0 ml. of sterile distilled water to the contents of one ampule and letting it stand for about one-half hour. The slightly opalescent solution thus obtained is stable for a practically unlimited period if kept in a sterile closed tube at 4 to 6°C. When used in the Reiter protein complement fixation test, this solution must be diluted 1:80 with physiologic salt solution.

b. Other reagents needed for the Reiter protein complement fixation (RPCF) test include guinea pig complement, glycerinized anti-sheep hemolysin, a 2% suspension of washed pooled sheep cells, physiological saline and Reactive syphilitic control serum. These items are prepared by the same methods which are explained in full detail under "Kolmer Complement Fixation Test." (See section III, paragraph E-2 - Stock Reagents, page 37).

3. Complement and Hemolysin Titrations:

The following will be useful to those already familiar with the Kolmer complement fixation technique.

a. Perform the two titrations simultaneously in the same rack.

b. Place 10 tubes (12 x 75 mm.) numbered 1 to 10 in one side of the rack for the hemolysin titration and 6 tubes (12 x 75 mm.) in the other side of the rack for the complement titration.

c. Place 10 tubes labeled 1 to 10 in another rack for the preparation of hemolysin dilutions. Use 13 x 100 mm. or 15 x 85 mm. test tubes.

d. Prepare a 1:1000 dilution of hemolysin by placing 4.5 ml. of saline

solution in the first tube of the rack for hemolysin dilutions and add 0.5 ml. of 1:100 stock hemolysin dilution. Mix well and add 0.5 ml. to tubes 2, 3, 4 and 5.

e. Add the required amount of saline solution to tubes 2 through 10 and complete the hemolysin dilutions by mixing and transferring as follows:

Table 20.

Tube Identification		Saline	Process	Final hemolysin Dilution
1st	1,000	0.0	None	1:1000
2nd	2,000	0.5	Mix	1:2000
3rd	3,000	1.0	Mix; transfer 0.5 to tube 6	1:3000
4th	4,000	1.5	Mix; transfer 0.5 to tube 7	1:4000
5th	5,000	2.0	Mix; transfer 0.5 to tube 8	1:5000
6th	6,000	0.5	Mix; transfer 0.5 to tube 9	1:6000
7th	8,000	0.5	Mix: transfer 0.5 to tube 10	1:8000
8th	10,000	0.5	Mix	1:10000
9th	12,000	0.5	Mix	1:12000
10th	16,000	0.5	Mix	1:16000

f. Transfer 0.1 ml. of each hemolysin dilution to the correspondingly numbered 12 x 75 mm. hemolysin titration tubes in the hemolysin and complement titration rack.

g. Prepare a 1:50 dilution of complement by adding 0.1 ml. of guinea pig serum to 4.9 ml. of saline solution and mixing well. Use a 0.1 ml. or 0.2 ml. pipet.

h. Pipet 0.1 ml. of 1:50 complement dilution into each of the 10 tubes of the hemolysin titration.

i. Add the following amounts of 1:50 complement dilution and saline to the complement titration tubes:

Tube	Complement 1:50 (ml.)	Saline (ml.)
1	0.05	0.45
2	0.10	0.4
3	0.15	0.35
4	0.20	0.3
5	0.25	0.25
6	0.30	0.2

j. Add 0.4 ml. of saline solution to each of the 10 tubes of the hemolysin titration.

k. Add 0.1 ml. of 2% sheep red cell suspension to each tube of the hemolysin titration.

l. Shake each tube of the hemolysin titration to insure even distribution of cells and place the rack containing the two titrations in the 37°C. water bath for 1 hour.

m. Remove rack from the water bath and read hemolysin titration. The unit of hemolysin is the highest dilution that gives complete sparkling hemolysis. Hemolysin for the complement titration and test proper is diluted so that 2 units are contained in 0.1 ml.

n. Prepare a quantity of diluted hemolysin, containing 2 units per 0.1 ml., sufficient for the complement titration as follows:

Table 21.

Dilution containing 1 unit per 0.1 ml.	Dilution containing 2 units per 0.1 ml.	To prepare 2 units dilution, mix 1:1000	
		Hemolysin (ml.)	Saline (ml.)
1:4000	1:2000	0.1	1.9
1:5000	1:2500	0.1	2.4
1:6000	1:3000	0.1	2.9
1:8000	1:4000	0.1	3.9
1:1000	1:5000	0.1	4.9
1:1200	1:6000	0.1	5.9
1:1600	1:8000	0.1	7.9

o. Add 0.1 ml. of diluted hemolysin (containing 2 units of hemolysin) to each of the six tubes of the complement titration.

p. Add the contents of 7th tube to the complement titration and place in a 0.6 ml. of saline solution. This serves as the cell control tube.

q. Add 0.1 ml. of 2% sheep red cell suspension to each of the complement titration tubes and to the cell control tube (tube 7).

r. Shake each tube of the complement titration to insure even distribution of cells and return to the 37°C. water bath for 1 hour. The completed complement titration is shown in following table.

Table 22. Completed Complement Titration

Tube No.	Complement 1:50	Saline Solution		Hemolysin	Sheep Cell suspension 2%	
	(ml.)	(ml.)		(ml.)	(ml.)	
1	0.05	0.45	37°C. water bath for one hour	0.1	0.1	37° water bath for 1 hr.
2	0.1	0.4		0.1	0.1	
3	0.15	0.35		0.1	0.1	
4	0.2	0.3		0.1	0.1	
5	0.25	0.25		0.1	0.1	
6	0.3	0.2		0.1	0.1	
7	None	0.6		None	0.1	

s. Remove the rack from the water bath and read the complement titration. The smallest amount of complement giving complete sparkling hemolysis is the exact unit. For conducting the tests two exact units are employed. This dose of 2 exact units should be contained in 0.2 ml. of the proper dilution as follows:

Table 23.

Exact Unit	Two Exact Units	Dose of Two Exact Units
0.10 ml.	0.2 ml.	0.2 ml. of 1:50 dilution
0.15 ml.	0.3 ml.	0.2 ml. of 1:33 dilution
0.20 ml.	0.4 ml.	0.2 ml. of 1:25 dilution
0.25 ml.	0.5 ml.	0.2 ml. of 1:20 dilution
0.30 ml.	0.0 ml.	0.2 ml. of 1:17 dilution

4. Reiter Qualitative Tests with Serum and Spinal Fluid:

a. Arrange test tubes in wire racks so that there are two tubes for each serum or spinal fluid to be tested. Control serum of graded reactivity should be included. Number the first row of tubes to correspond to the serum or spinal fluid to be tested. Three additional test tubes are included for reagents controls.

b. Add the following amounts of saline solution to the three reagent control tubes:

Antigen control ..... 0.2 ml. saline solution
Hemolytic system control ..... 0.3 ml. " "
Corpuscle control ..... 0.6 ml. " "

c. For qualitative testing with serum add 0.8 ml. of saline solution to the first row and 0.1 ml. to the second row. Add 0.2 ml. of inactivated serum to the proper tube in the front row, mix, withdraw 0.8 ml., transfer 0.2 ml. to the proper tube in the second row and discard 0.6 ml. Thus 0.04 ml. of serum are contained in both tubes. The second row tubes are serum control tubes.

d. For qualitative testing with spinal fluid pipet 0.1 ml. of saline solution into each tube in the first row and 0.2 ml. into each tube in the second row. Add 0.1 ml. of spinal fluid to each row.

e. Pipet 0.2 ml. of control serum or serum dilution into the respective first and second row tubes and add 0.1 ml. of saline solution to the tubes of the second row.

f. Add 0.1 ml. of antigen dilution to the first tube of each test (serum, spinal fluid or control serum) and to the antigen control tube.

g. Shake each rack well and allow to stand at room temperature for 10 to 15 minutes.

h. Prepare diluted complement during this interval. The amount needed is equivalent to 0.2 ml. for each tube of the test plus a slight excess. Complement serum should be diluted with cold saline, i.e., 6° to 8°C. The volume of complement serum to be diluted is determined by the amount of diluted complement necessary for the test. Dividing the number of millileters of diluted complement needed by the complement dilution factor (two exact units) will give the number of milliliters of complement serum needed.

i. Add 0.2 ml. of diluted complement (containing two exact units) to all tubes of the serum and spinal fluid tests, control serum and to the antigen and hemolytic system control tubes.

j. Mix the contents of the tubes by shaking the racks well and place in the refrigerator at 6° to 8°C. for 15 to 18 hours.

k. Prepare the volume of diluted hemolysin needed for the test proper, allowing 0.1 ml. (containing 2 units) for each tube. (Prepare a slight excess.) The following formula may be used for calculating the amounts of 1:100 hemolysin solution and diluent required to prepare the needed volume of diluted hemolysin. $\frac{100}{\text{Hemolysin titration}} \times \text{Volume of diluted hemolysin needed} = \text{ml. of 1:100 hemolysin required.}$ If one needed 12 ml. of 1:4000 hemolysin dilution: $\frac{100}{4000} \times 12 = 0.3$. Then 0.3 ml. of 1:100 hemolysin dilution plus 11.7ml.saline solution would give the required 12 ml. of 1:4000 hemolysin dilution.

l. Remove racks of tubes from the refrigerator at regular intervals and place immediately in the 37°C. water bath for 10 minutes. The interval will be determined by the length of time necessary to add hemolysin and sheep cell suspension to each rack.

m. Remove each rack from the water bath and add 0.1 ml. of the diluted hemolysin to all tubes of the test except the corpuscle control tube.

n. Add 0.1 ml. of the 2% sheep red cell suspension (prepared the previous

day) to all tubes. The 2% sheep red cell suspension should be agitated occasionally to secure an even suspension of cells during the period when this reagent is being added to the tests.

o. Mix the contents of the tubes thoroughly by shaking each rack before returning it to the 37°C. water bath for the secondary incubation. Examine the controls at 5 minute intervals. The period of secondary incubation will be determined by the length of time necessary to reproduce the predetermined reactivity pattern of the control serums.

p. Remove each rack of tubes from the water bath at the end of the secondary incubation period. Record the degrees of hemolysis as described under "Preparation of Reading Standards" and "Reading and Reporting Test Results" except in those instances where inhibition of hemolysis is noted in the control tube. All serums and spinal fluids showing inhibition of hemolysis in the control tube following secondary incubation not exceeding one hour should be considered anticomplementary. At the end of this period, these tubes are removed from the water bath, and test readings (including the control tubes) are recorded.

NOTE: Secondary Incubation Time: The sensitivity and reproducibility of the RPCF test are controlled by use of a Reactive control serum of known reactivity and in this respect the test differs from the conventional Kolmer technique. The secondary incubation period is dependent upon the time necessary for the Reactive control serum to produce a predetermined pattern of reactivity. RPCF tests should not be performed unless one includes a reactive control serum of established reactivity for determining the duration of secondary incubation. A pattern of 4+, 2+, and non-reactive can be established with proper dilutions of any Reactive Syphilitic Serum.

q. The complete outline of qualitative testing with serum and spinal fluid is as follows:

Table 24. Qualitative Testing Outline

Tube	Serum (1:5 dilution)	Saline Solution	Antigen		Complement 2 exact units		Hemolysin 2 units	Sheep Cell Suspension 2%	
	(ml.)	(ml.)	(ml.)		(ml.)		(ml.)	(ml.)	
1	0.2	None	0.1	Shake rack well. Allow to stand at room temp. 10 to 15 minutes.	0.2	Shake rack well. Primary incubation 15 to 18 hrs. at 6°-8°C. followed by 10 min. in 37°C. water bath.	0.1	0.1	Shake rack well. Secondary incubation in 37°C. water bath
2	0.2	0.1	None		0.2		0.1	0.1	
	Spinal Fluid (ml.)								
1	0.1	0.1	0.1		0.2		0.1	0.1	
2	0.1	0.2	None		0.2		0.1	0.1	
	Antigen Control								
	None	0.2	0.1		0.2		0.1	0.1	
	Hemolytic Control								
	None	0.3	None		0.2		0.1	0.1	
	2% Sheep Cell Control								
	None	0.6	None		None		None	0.1	

5. Reiter Quantitative Test with Serum and Spinal Fluid:

a. Place test tubes in wire racks, allowing 8 tubes for each serum and 6 tubes for each spinal fluid to be tested. Include reagent controls and control serums of graded reactivity.

b. For each serum pipet 0. 8 ml. of saline solution into tube 1 and 0. 2 ml. into tubes 2, 3, 4, 5, 6, and 7; add 0. 1 ml. to tube 8 (Control).

c. For each spinal fluid pipet 0. 3 ml. of saline solution into tube 1 and 0. 2 ml. into tubes 2, 3, 4, and 5.

d. Pipet the indicated amount of saline solution into the following reagent control tubes:

Antigen Control ..... 0. 2 ml.
Hemolytic control ..... 0. 3 ml.
Sheep Cell Control 2% ..... 0. 6 ml.

e. For each serum proceed as follows:

Table 25. Serum Process

Tube No.	Process	Serum Dilution
1.	Add 0. 2 ml. of inactivated serum. Mix and transfer 0. 2 ml. to tube 8 (Control) and to tube 2. Discard 0. 4 ml.	Undiluted
2.	Mix. Transfer 0. 2 ml. to tube 3.	1:2
3.	Mix. Transfer 0. 2 ml. to tube 4.	1:4
4.	Mix. Transfer 0. 2 ml. to tube 5.	1:8
5.	Mix. Transfer 0. 2 ml. to tube 6.	1:16
6.	Mix. Transfer 0. 2 ml. to tube 7.	1:32
7.	Mix. Discard 0. 2 ml.	1:64
8.		Undiluted (Control)

f. For each spinal fluid proceed as follows:

Table 26. Spinal Fluid Process

Tube No.	Process	Spinal Fluid Dilution
1.	Add 0. 3 ml. of spinal fluid. Mix and transfer 0. 2 ml. to tube 6 (Control) and tube 2	Undiluted
2.	Mix. Transfer 0. 2 ml. to tube 3.	1:2
3.	Mix. Transfer 0. 2 ml. to tube 4.	1:4
4.	Mix. Transfer 0. 2 ml. to tube 5.	1:8
5.	Mix. Discard 0. 2 ml.	1:16
6.		Undiluted (Control)

g. Add 0.1 ml. of diluted antigen to the first seven tubes of each serum test, to the first five tubes of each spinal fluid test, and to the antigen control tube. Shake the racks to mix thoroughly.

h. Allow racks to stand at room temperature for 10 to 15 minutes.

i. Complete the tests as indicated in paragraphs 8 through 16 of the section on qualitative testing of serum and spinal fluid.

j. The outline of quantitative tests with serum and spinal fluid is as follows:

Table 27.

Tube No.	Serum (in 0.2 ml.)	Antigen (ml.)		Complement (2 exact units) (ml.)		Hemolysin (2 units) (ml.)	Sheep Cell Suspension (2%) (ml.)	
1	0.04 (Undiluted)	0.1	Shake racks well, allow to stand at room temperature 10 to 15 minutes.	0.2	Shake rack well. Primary incubation 15 to 18 hours at 6° to 8°C. Followed by 10 minutes in 37°C. water bath	0.1	0.1	Shake rack well, secondary incubation in 37°C. water bath.
2	0.02 (1:2)	0.1		0.2		0.1	0.1	
3	0.01 (1:4)	0.1		0.2		0.1	0.1	
4	0.005 (1:8)	0.1		0.2		0.1	0.1	
5	0.0025 (1:16)	0.1		0.2		0.1	0.1	
6	0.0013 (1:32)	0.1		0.2		0.1	0.1	
7	0.0006 (1:64)	0.1		0.2		0.1	0.1	
8	0.04 (Undiluted control)	None		0.2		0.1	0.1	
	Spinal Fluid in (0.2 ml.)							
1	0.1 (Undiluted)	0.1		0.2		0.1	0.1	
2	0.05 (1:2)	0.1		0.2		0.1	0.1	
3	0.025 (1:4)	0.1		0.2		0.1	0.1	
4	0.0125 (1:8)	0.1		0.2		0.1	0.1	
5	0.0063 (1:16)	0.1		0.2		0.1	0.1	
6	0.1 (Undiluted control)	0.1		0.2		0.1	0.1	
	Antigen Control 0.2 ml. saline solution	0.1		0.2		0.1	0.1	
	Hemolytic Control. 0.3 ml. saline solution	None		0.2		0.1	0.1	
	2% Sheep Cell Control. 0.6 ml. saline solution	None		None		None	0.1	

6. Preparation of Reading Standards:

a. Until considerable experience has been gained in the reading of reactions, it is advisable to prepare scales showing the appearance of 4+, 3+, 2+, 1+, $\pm$, and - complement fixation reactions.

b. Hemoglobin solution used for the preparation of reading standards is obtained by saving titration tubes or control tubes of the current days tests that show complete hemolysis. Save 6 or 6 tubes and pool them; 2.87 ml. is the exact amount of hemoglobin solution needed.

c. Heat tubes of hemoglobin solution in the 56°C. water bath for 5 minutes.

d. Prepare a 1:7 dilution of 2% suspension to 2.0 ml. of saline.

e. Prepare reading standards by mixing hemoglobin solution and cell suspension in the following proportions:

Table 28. Reading Standards

1:7 Sheep RBC Suspension (ml.)	Hemoglobin Solution (ml.)	Equivalent Complement fixation %	Record
0.7	None	100	4+
0.35	0.35	50	3+
0.18	0.63	25	2+
0.07	0.63	10	1+
0.03	0.67	5	$\pm$
None	0.7	0	-

7. Reading and Reporting Test Results:

a. All serum and spinal fluid controls should show complete hemolysis.

b. Estimate the individual tube readings by comparison with the reading standards at the end of the secondary incubation period and record the degree of complement fixation noted, except for those specimens showing inhibition of hemolysis in the control tube.

c. Read the tubes that have been returned to the 37°C. water bath for a full hour's secondary incubation, estimating and recording the degree of complement fixation of each tube and control tube by comparison with the reading standards.

d. Report the results of qualitative tests according to the following examples.

Test tube reading	Control tube reading	Report
4+	-	Reactive
3+	-	Reactive
2+	-	Reactive
1+	-	Reactive
±	-	Weakly Reactive
-	-	Non-Reactive
4+	4+	Anticomplementary
4+	3+	Anticomplementary
4+	2+	Weakly Reactive
4+	1+	Reactive

Test tube reading	Control tube reading	Report
3+	3+	Anticomplementary
3+	2+	Anticomplementary
3+	1+	Weakly Reactive
3+	±	Reactive
2+	2+	Non-Reactive
2+	1+	Non-Reactive
2+	±	Weakly Reactive
1+	1+	Non-Reactive
±	±	Non-Reactive

e. Quantitative tests are reported in terms of the highest dilution giving a Reactive result (1+, 2+, 3+, or 4+) as illustrated below:

Serums or Spinal Fluid

Undiluted	Dilutions						Report
	1:2	1:4	1:8	1:16	1:32	1:64	
4	3	1	-	-	-	-	Reactive, 1:4 dilution, or 4 dils.
4	-	-	-	-	-	-	Reactive (4+), undil., or 1 dil.
4	4	3	2	-	-	-	Reactive, 1:8 dilutions, or 8 dils.
4	4	4	4	4	1	-	Reactive, 1:32 dilution, or 32 dils.
3	1	-	-	-	-	-	Reactive, 1:2 dilution, or 2 dils.
1	-	-	-	-	-	-	Reactive (1+), undil., or 1 dil.
±	-	-	-	-	-	-	Weakly Reactive
-	-	-	-	-	-	-	Nonreactive
4	4	4	4	4	4	4	Retest 1:64 and higher dilutions.

f. Only qualitative reports are made when the full-hour incubation at 37°C. is required. Report the results of the quantitative tests in accordance with the following examples when the full-hour incubation at 37°C. is required.

Test tube reading							Control tube reading	Report
4	4	4	4	4	2	-	4+	Anticomplementary
4	4	3	-	-	-	-	2+	Reactive
4	4	1	-	-	-	-	1+	Reactive
3	2	-	-	-	-	-	+	Reactive
4	4	1	-	-	-	-	3+	Weakly Reactive
3	2	-	-	-	-	-	1+	Weakly Reactive
2	1	-	-	-	-	-	±	Weakly Reactive
3	-	-	-	-	-	-	±	Reactive
1	-	-	-	-	-	-	±	Nonreactive
1	-	-	-	-	-	-	1+	Nonreactive
2	-	-	-	-	-	-	1+	Nonreactive
2	-	-	-	-	-	-	2+	Nonreactive

8. References

Public Health Report 72:335, 1957

Manual of Serologic Tests for Syphilis. Dept. H.E.W., Public Health Service publication No. 411 (1959 revision). Supt. of Documents, Govt. Printing Office, Washington, D.C.

Approved Laboratory Technic. Kolmer, Spaulding and Robinson, Appleton-Century Crofts, Inc., New York, N.Y., 5th edition, 1951.

H. TREPONEMA PALLIDUM IMMOBILIZATION TEST

1. Introduction and History:

Many investigators have explored the theory that a specific antibody produced against virulent Treponema pallidum is present in the serum of patients with syphilis. It was hypothesized that this antibody was associated with the tissue instead of being in the blood serum. The existence of the antibody was not proved because it was impossible to keep the treponeme alive in vitro for an appreciable length of time. The natural death rate of the treponeme progressed logarithmically over a period of 6 hours, with or without the addition of syphilitic serum after which the majority of the spirochetes were dead. Various investigators tried to solve this problem of survival but met with little success.

In 1947, Nelson, while conducting experiments directed toward culturing T. pallidum in vitro, discovered the immobilizing antibody. During the year that followed, he attempted to define some of the physical and chemical factors which influenced the organism's survival in vitro. These preliminary experiments were performed with virulent T. pallidum (Nichol's strain) obtained from syphilitic rabbit testes emulsified in the Waring blender. The concentration of treponemes used was 5 to 10 x 10^6 organisms per mililiter in a tissue-free suspension. No multiplication was observed and survival was limited to a half-life of 96 hours; however, these experiments did show the beneficial effect of carbon dioxide and the detrimental effect of oxygen.

Using the medium, techniques, and knowledge thus far gained, it was possible to consistently obtain survival of 50% of the organisms for 6 to 8 days. The fact that a medium was now available made possible an approach to the study of the immunology of syphilis. In July, 1948, Nelson made the first antibody run, which was the fore-runner of what we now know as the Treponema pallidum immobilization (TPI) test. Mayer contributed many ideas on the interpretation and application of antibody reaction. These were based on his previous work on the hemolytic antibody. During the latter part of 1949 and early 1950, Rice and Nelson isolated a survival factor for T. pallidum from beef serum. It was also found that the antibody was related to immunity.

a. Preliminary Investigations Leading to the Discovery of TPI Antibody:

The first experiments were set up to determine the beneficial action of various metabolites. Since the fact had already been established that the organism was a strict anaerobe, experiments were performed in oxygen-free atmospheres. Nitrogen, carbon dioxide, and hydrogen were assayed. One of these experiments was performed in an atmosphere composed of 95% nitrogen and 5% carbon dioxide to demonstrate the beneficial effect of carbon dioxide on survival. A beneficial effect of inorganic phosphate in combination with the carbon dioxide-bicarbonate system is also known and effective. The optimum concentration, however, was not determined.

Since it was postulated that the combination of carbon dioxide and certain intermediates in carbohydrate metabolism might prove beneficial to the survival of T. pallidum, the effect of citrate, succinate, and pyruvate were investigated. Only pyruvate proved to be beneficial.

The next step was to determine the effect of the sulfhydryl compounds on the survival of the treponeme. One-thousandth molar solutions of glutathione, cysteine, and ascorbic acid in .85% saline were tested individually and in various combinations for their effect on the survival of spirochetes obtained from the extraction of rabbit testes. A definite inhibitory effect of ascorbic acid was noted. The beneficial effect of glutathione and cysteine was not observed until after 96 hours of incubation. Their combination in equimolar concentrations appeared more stimulatory than either used individually, since 76% of the organisms remained motile after 96 hours; but in the absence of these compounds only 30% survived the 96 hour incubation period.

The next addition to the group of necessary metabolites was ultrafiltrate of beef serum. The active component of the serum ultrafiltrate was isolated in pure crystalline form by Rice and Nelson. In the presence of ultrafiltrate of beef serum 48% of the organisms remained motile for five days, whereas in the absence of ultrafiltrate, survival dropped to zero per cent in only two days.

With survival increased to a period of several days, it was not possible to demonstrate the presence and action of the immobilizing antibody as well as the necessity for complement in the system. This was done by exposing T. pallidum to a normal and a syphilitic serum under the same conditions with and without complement.

In the absence of complement there is no appreciable difference in the number of organisms that survived either in normal or syphilitic serum. However, there was a marked difference in the number of organisms surviving when samples of the same sera were incubated for the same period of time in the presence of complement. In the normal serum a high percentage of the organisms remained motile during the 24 hour period, while in the syphilitic serum the survival dropped as low as 2% after a lapse of only 12 hours. This evidence suggests that an antibody which causes the immobilization of T. pallidum in the presence of complement exists in syphilitic serum but does not exists in normal serum.

It has been shown that there is a difference between immobilizing antibody and Complement Fixing reagin in untreated experimental syphilis. The complement fixing titre reaches its peak the twelfth month following infection, then starts a gradual decline, leveling off at a low titre after the twenty-fourth month, and eventually drops to zero. The TPI antibody titre, however, does not reach its peak until the tenth month and then remains at a high level. It is thought that this high level persists for life.

The implication that TPI antibody and Complement fixing reagin were completely different substances, was confirmed by taking a reactive serum of known titre and absorbing out the Complement fixing reagin by flocculation with Eagle antigen. The reagin titre of the serum was 16 dils. After one absorption the titre dropped from 16 to 2 dils. and was negative after the second absorption. The TPI titre remained at a high titre throughout, confirming the fact that TPI antibody is separate and distinct from Complement fixing reagin.

b. The Navy Program:

In April, 1951, a program was approved by the Bureau of Medicine and Surgery whereby all personnel in the U.S. Navy suspected of having latent syphilis, on the basis of a reactive or weakly reactive standard serological test for syphilis (reagin test), were required to have serum submitted for a TPI Test.

Certain aspects of the fundamental immunological role of TPI antibody are still unresolved. Since the specific occurrence of TPI antibody in a syphilitic infection had been established in sufficient clinical trials, it was decided to use the TPI test as a diagnostic criterion for latent syphilis. Accordingly, persons showing repeated reactive tests were diagnosed as having, or having had syphilis, and those with repeated nonreactive tests as never having had the infection.

In addition to the above considerations, it was felt the performance of the TPI test in the Navy would serve as a means of evaluating the practicability of this test. Therefore a careful record was to be kept in regard to (1) the condition of sera received from the various naval units, and (2) the performance of the test by naval technicians, with reference to their ability to cope with the numerous problems of the rather complicated test procedure.

2. Eligibility of Patients:

The criteria for submitting serum, as set forth by the United States Navy, Bureau of Medicine and Surgery, are:

a. Two or more reactive or weakly reactive standard reagin tests;
b. No history of, previous diagnosis of, or treatment for syphilis;
c. No present signs or symptoms of primary, secondary, or late symptomatic syphilis.

3. Shipment and Handling of Sera for TPI Testing at U.S. Naval Medical School, National Naval Medical Center, Bethesda , Maryland, 20014.

a. A sample of 10 mls. of sterile serum should be sent at the earliest possible date, via air, whenever feasible. Include name of activity and patient's name, rate and service number. Label mailing carton "FOR TPI TEST."

b. The following precautions in obtaining specimens will eliminate many unsatisfactory results.

(1) Past experience has shown that where the donor has received injections of antibiotics, other than penicillin, within a month, or received oral antibiotics within a week of the drawing of a blood sample, unreliable results were obtained.

(2) Glassware used in connection with obtaining and forwarding sera for TPI tests must be acid cleaned (Potassium Dichromate Cleaning Solution). After cleaning, each piece of glassware must be individually rinsed 20 times under running tap water and 10 times under running distilled water, then dried, plugged and sterilized.

(3) Draw 20 ml. of blood, allow to clot, then separate serum aseptically. Place serum in an acid cleaned test tube and stopper with a paraffin coated cork. Uncoated cork stoppers or rubber stoppers are unsatisfactory. Test tube 16 x 125 mm. with screw cap (Federal Supply Stock) may also be used, or blood may be drawn in a vacuum tube, such as a B-D vacutainer, providing the rubber stopper is immediately replaced with a coated cork stopper.

(4) It is highly recommended that a fasting blood sample be drawn, since the presence of post-prandial fats in the serum interfere with technical performance of the test.

(5) No chemical preservatives, such as merthiolate, sodium azide, etc., should be added to serum.

(6) Under no circumstances should whole blood be submitted for testing.

NOTE: It is requested that a label bearing the name of the patient and the Naval Activity be affixed to the test tube of sera submitted for testing. This will minimize errors in handling the specimen enroute.

(7) In addition to all of the above information, a properly completed Standard Form DD-876 is submitted with each specimen.

4. Material for the Test:

It is necessary to stress the fact that aseptic technique is maintained throughout the entire performance of the test procedure.

a. Spirochetes: The Nichols pathogenic strain of Treponema pallidum is maintained as source material for the TPI test.

b. Rabbits: Adult male rabbits with testes 1 to 1 1/2 inches; must be clean, healthy, antibiotic-free. Preferably no black rabbits. The rabbits should weigh approximately 5 to 6 kilograms. They are caged in an air-conditioned room at 10 to 20°C. temperature.

5. Preparation of Basal Medium for TPI Test

The basal medium is made just prior to each use from separate stock solutions of primary constituents. The latter are prepared as follows:

a. Bovine Plasma, Fraction V (Armour) - 5% solution:

Dissolve 20.0 Gms. of Fraction V in 350 ml. of 0.65% NaCl solution. Add sufficient 1.0 ml. NaOH to adjust pH to 7.0. Then add sufficient 0.85% NaCl to make 400 ml. of solution. Sterilize by Selas Micro-Porous Filters and store at 5°C.

b. Phosphate buffer solution - pH 7.1:

Reagent "A" Na^2PO^4 0.1 ml. Dissolve 7.10 Gm. of Na^2PO^4 in triple distilled water sufficient to make 500 ml. of solution.

Reagent "B" KH^2PO^4 0.15 ml. Dissolve 10.21 Gm. of KH^2PO^4 in triple-distilled water sufficient to make 500 ml. of solution.

Prepare a solution for use by mixing 120 ml. of reagent "A" with 20 ml. of reagent "B". Sterilize by filtration (Corning U.F.) and store at 5°C.

c. Sodium Bicarbonate - 1.26% solution:

Dissolve 1.26 Gm. of $NaHCO_3$ in triple distilled water sufficient to make 100 ml. of solution. Sterilize by filtration (Corning U.F.) and store at 5°C. MAKE FRESH BIWEEKLY.

d. Sodium Chloride- 0.8% solution:

Dissolve 1.70 Gm. of NaCl in triple distilled water sufficient to make 200 ml. of solution, sterilize by filtration (Corning U.F.) and store at 5°C.

e. Sodium Thioglycollate - 1.50% solution:

Dissolve 0.75 Gm. of sodium thioglycollate in triple-distilled water sufficient to make 50 ml. of solution. Sterilize by filtration (Corning U.F.), Pipet 3.0 ml. of the solution into sterile 13 x 100 mm. test tube, cap, and store at -20°C. NOTE: MAKE FRESH BIWEEKLY.

f. Cysteine HCl - 0.63 solution:

Dissolve 0.630 Gm. of cysteine HCl in 0.85% NaCl solution sufficient to make 100 ml. of solution. Sterilize by filtration (Corning U.F.). Pipet 3.0 ml. into sterile 13 x 100 mm. test tubes, cap and store at -20°C. NOTE: MAKE FRESH BIWEEKLY.

g. Glutathione - 1.23% solution:

Dissolve 1.23 Gm. of glutathione in 0.85% NaCl solution sufficient to make 100 ml. of solution. Sterilize by filtration (Corning U.F.). Pipet 3.0 ml. into sterile 13 x 100 mm. tubes, cap and store at -20°C. NOTE: MAKE FRESH BIWEEKLY.

h. Sodium Pyruvate - 1.0% solution:

Dissolve 0.5 Gm. of sodium pyruvate in 0.85% NaCl solution sufficient to make 50 ml. of solution. Sterilize by filtration (Corning U.F.). Pipet 2.0 ml. into sterile 13 x 100 mm. test tubes, cap, and store at -20°C. NOTE: MAKE FRESH BIWEEKLY.

i. Ultrafiltrate bovine serum:

Remove from bottle aseptically and place in evacuation flask. Place under CO_2 and store at 5°C.

j. Composition from stock solutions:

When an extraction is to be done, prepare the basal medium from the stock solution as follows:

Table 29. Preparation of Basal Medium

	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)
Serum Fraction V	5.0	10.0	20.0	30.0	40.0
Phosphate Buffer	1.6	3.13	6.26	9.39	12.52
Sodium thio	0.3	0.60	1.20	1.80	2.40
Glutathione	0.3	0.63	1.26	1.89	2.52
Cysteine	0.3	0.63	1.26	1.89	2.52
Sodium Pyruvate	0.2	0.31	0.62	0.93	1.24
Sodium Bicarb	0.6	1.13	2.26	3.39	4.52
0.85% NaCl	1.2	2.32	4.64	6.96	9.28
UFBS	0.6	1.25	2.50	3.75	5.00
	10.0 ml.	20.00 ml.	40.00 ml.	60.00 ml.	80.00 ml.

6. Antigen Preparation:

The testicles of a rabbit that has developed a typical orchitis some five to ten days after injection of live Treponemes are utilized in preparing this antigen.

a. The rabbit is sacrificed by injecting air into the marginal vein of the ear.

b. Each testicle is clamped off with an Oschner forceps and is surgically removed with a scalpel by cutting beneath the forceps.

c. The entire scrotum and forceps are placed in a Petri dish and, using alcohol sponge, scalpel, rat-tooth thumb forceps and scissors, the entire testicles are aseptically removed from the sac. They are transferred to a second sterile Petri dish.

d. Hemisection by one longitudinal incision of the testicle, but leave the halves held together by the outer testicular membrane. Mascerate the inner testicular tissue with scalpel and scissors. Rinse with 0.85% saline and transfer to an extraction flask containing 10 ml. of Basal Medium.

e. The flask is evacuated and flushed three times with Nitrogen and CO^2 mixture and left under gas.

f. The flask is allowed to shake on the Kahn shaker for 10 to 15 minutes and then microscopically examined under darkfield illumination. The number of Treponemes per high-dry field is determined. The supernatant fluid is poured off and centrifuged at 2200 rpm for 10 minutes.

At this point an additional 5 to 10 ml. of Basal Media is added, the flask regassed and put back on the shaker for as much as 1 to 3 hours. This second extraction is for inoculation of additional rabbits.

g. The clear fluid, free of RBC's and debris, is diluted with Basal Media to prepare an antigen containing not less than 7 nor more than 13 Treponemes per high-dry field. The per cent motility should be determined by examining 50 Treponemes under darkfield illumination. The number motile is multiplied by two and should approach 100%.

h. The antigen is now ready for immediate use.

7. Preparation and Preservation of Guinea Pig Complement:

Aseptically, collect blood from at least six healthy, preferably male, adult guinea pigs. Allow blood to clot under refrigeration. Centrifuge at 5°C. and pool the sera. Dispense in two or three mls. amounts in sterile screw cap test tubes. Quick freeze in an alcohol-dry ice bath and store at -40°C.

a. For use in the TPI test this Complement must have a titer of at least 1:37 in the Kolmer Complement Titration.

8. Procedure for TPI Test

a. All unknown sera plus a known negative serum, 1.0 ml. of 1:10 dilution of a positive control serum and sufficient complement are placed in the 56°C water bath for 30 minutes.

b. Penicillinase is added to each unknown sera (0.05 ml. per 1.0 ml. of serum) and allowed to stand 30 minutes in room temperature.

c. Serial dilutions are made from the 1:10 positive control so that the final dilutions will be 1:20, 1:40, 1:80, 1:160, 1:320. These dilutions are good for one week.

d. Set up and number a rack of 13 x 100 mm. sterile, cotton and gauze stoppered test tubes sufficient for nine control tubes plus three tubes for each unknown serum to be tested. Circle the number of the tube #2, #4, and the first tube of each series of unknown (i.e., #10, #13, #16, etc.)

e. Pipet: into tube #1, 0.1 ml. of 0.85% saline;
into tubes #2 and #3, 0.05 ml. of negative serum;

f. Tubes #4 through #9 are the positive serum controls and are pipetted in the following manner: #4 and #5 - 0.05 ml. 1:20 dilution; #6 - 0.05 ml. 1:40 dilution; #7 - 0.5 ml. 1:80 dilution; #8 - 0.5 ml. 1:160 dilution; and #9 - 0.5 ml. 1:320 dilution.

g. Into triplicate tubes pipet 0.05 ml. of unknown serum. (The first serum into #10, #11, and #12, the second into #13, #14, and #15, etc.)

h. Pipet 0.05 ml. of previously inactivated complement into each tube with a circled number, e.g., #2, #4, and the first tube of each series of unknowns.

i. At this point the tests are left at room temperature while the antigen is prepared.

j. During the preparation of the antigen and immediately prior to its addition, pipet 0.05 ml. of active complement to each tube that is not circled (with the exception of tube #1), i.e., #3, #5, #6, #7, #8, #9, and the second and third tubes of each unknown series.

k. Remove all cotton stoppers and add 0.4 ml. of antigen to each tube.

i. All tubes are placed into a Brewer Anaerobic Jar and sealed. (The air is evacuated and the jar is flushed with Nitrogen and CO^2 mixture for a total of three times and left under this gas for 20 hours at 35°C.)

m. After 20 hours incubation, each tube is examined microscopically under darkfield illumination and the per cent motility determined by examining fifty Treponemes and multiplying the number motile by two. The per cent containing active complement is the per cent specifically immobilized.

n. Test each tube of the run for the presence of Residual Complement. This is done in the following manner. Prepare a 2% suspension of thrice washed sheep RBC's in Kolmer Saline. Sensitize by adding equal volumes of 1:1000 Hemolysin and 2% Sheep Cells. Allow to stand at room temperature for at least ten minutes. Further dilute by adding 1.0 ml. of Kolmer Saline to each 4.0 ml. of sensitized cells.

Pipet 0.25 ml. of this mixture to each tube of the run and incubate in the 37°C. water bath for 30 minutes. Check for hemolysis.

9. Interpretation of Results:

a. The tubes numbered 1 through 9 are controls. Tube #1 is the saline control and should approach 100% motility. Tubes #2 and #3, are the negative control and the per cent specifically immobilized should not exceed 5%. Tubes #4 through #9, are the positive controls. The per cent specifically immobilized is determined for tubes #5 through #9, and the 50% end point as titer of the control serum determined.

b. The unknown sera are set up in three tubes. The first is with inactive complement and the remaining two are duplicates containing active complement. The first tube should approach 100% motility and if it is below 76% the serum is considered toxic. This is also known as Non-Specific Immobilization and is caused by some substance, other than antibody, within the serum that immobilizes the Treponemes.

c. The per cent specifically immobilized is determined for the second and third tubes. Since they are duplicates the per cent specifically immobilized should be approximately the same.

d. If upon doing the test for Residual Complement, complete hemolysis is observed in the tubes containing active Complement and no hemolysis is noted in the other tubes the following results may be reported. Per cent Specifically Immobilized: 0-20% Negative, 21-70% Weakly Reactive; 71-100% Reactive. All weakly reactive results should be repeated before reporting.

e. If any tube containing active complement does not show complete hemolysis in the test for Residual Complement, and the results are Negative or Weakly Reactive, this test is invalid and must be repeated.

Table 30

TREPONEMA PALLIDUM IMMOBILIZATION TEST

CALCULATION OF ORGANISMS SPECIFICALLY IMMOBILIZED (%)

% MOTILE IN TUBE CONTAINING COMPLEMENT

% MOTILE IN TUBE CONTAINING INACTIVE COMPLEMENT	%	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	86	88	90	92	94	96	98	100
	100	98	96	94	92	90	88	86	84	82	80	78	76	74	72	70	68	66	64	62	60	58	56	54	52	50	48	46	44	42	40	38	36	34	32	30	28	26	24	22	20	18	16	14	12	10	8	6	4	2	0
	98	98	96	94	92	90	88	86	84	82	80	78	76	73	71	69	67	65	63	61	59	57	55	53	51	49	47	45	43	41	39	37	35	33	31	29	27	25	22	20	18	16	14	12	10	8	6	4	2	0	
	96	98	96	94	92	90	88	85	83	81	79	77	75	73	71	69	67	65	62	60	58	56	54	52	50	48	46	44	42	40	38	35	33	31	29	27	25	23	21	19	17	15	13	10	8	6	4	2	0		
	94	98	96	94	91	89	87	85	83	81	79	76	74	72	70	68	66	64	62	60	57	55	53	51	49	47	45	43	40	38	36	34	32	30	28	26	24	21	19	17	15	13	11	9	6	4	2	0			
	92	98	96	93	91	89	87	84	82	80	78	76	74	72	70	67	65	63	61	59	57	54	52	50	48	46	43	41	39	37	35	33	30	28	26	24	22	20	17	15	13	11	9	7	4	2	0				
	90	98	96	93	91	89	87	84	82	80	78	76	73	71	69	67	64	62	60	58	56	53	51	49	47	44	42	40	38	36	33	31	29	27	24	22	20	18	16	13	11	9	7	4	2	0					
	88	98	95	93	91	89	86	84	82	80	77	75	73	70	68	66	64	61	59	57	55	52	50	48	45	43	41	39	36	34	32	30	27	25	23	20	18	16	14	11	9	7	5	2	0						
	86	98	95	93	91	88	86	84	81	79	77	74	72	70	67	65	63	60	58	56	53	51	49	47	44	42	40	37	35	33	30	28	26	23	21	19	16	14	12	9	7	5	2	0							
	84	98	95	93	91	88	86	83	81	79	76	74	71	69	67	64	62	60	57	55	52	50	48	45	43	40	38	36	33	31	29	26	24	21	19	17	14	12	9	7	5	2	0								
	82	98	95	93	90	88	85	83	80	78	76	73	71	68	66	64	61	59	56	54	51	49	46	44	41	39	36	34	32	29	27	24	22	20	17	15	12	10	7	5	2	0									
	80	97	95	92	90	87	85	82	80	77	75	72	70	67	65	62	60	57	55	52	50	47	45	43	40	38	35	33	30	28	25	23	20	18	15	13	10	8	5	3	0										
	78	97	95	92	90	87	85	82	79	77	74	72	69	67	64	62	59	56	54	51	49	46	44	41	39	36	33	31	28	26	23	21	18	15	13	10	8	5	3	0											
	76	97	95	92	89	87	84	82	79	76	74	71	68	66	63	60	58	55	53	50	47	45	42	39	37	34	32	29	26	24	21	18	16	13	11	8	5	3	0												
	74	97	95	92	89	86	84	81	78	76	73	70	68	65	62	59	57	54	51	49	46	43	40	37	34	31	29	26	23	21	19	16	14	11	8	5	3	0													
	72	97	94	92	89	86	83	81	78	75	72	69	67	64	61	58	56	53	50	47	44	42	39	36	33	31	28	25	22	19	17	14	11	8	6	3	0														
	70	97	94	91	89	86	83	80	77	74	71	69	66	63	60	57	54	51	49	46	43	40	37	34	32	29	26	23	20	17	14	11	9	6	3	0															
	68	97	94	91	88	85	82	79	76	74	71	68	65	62	59	56	53	50	47	44	41	38	35	32	29	26	24	21	18	15	12	9	6	3	0																
	66	97	94	91	88	85	82	79	76	73	70	67	64	61	58	55	51	48	45	42	39	36	33	30	27	24	21	18	15	12	9	6	3	0																	

REFERENCES:

Zinsser, H. and Hopkins, J.G.: Studies on Treponema pallidum and Syphilis. H. Spirochaeticidal Antibodies against Treponema pallidum. J. Exper. Med. 23: 323-328, 1916.

Kolmer, J.A., Broadwell, S., Jr., and Matsunami, T.: Agglutination of Treponema pallidum in Human Syphilis. J. Exper. Med. 24: 333-344, 1916.

Noguchi, H. and Akatsu, S.: Immunological Studies on Pure Cultures of Various Spirochetes. J. Exper. Med. 25: 765-788, 1917.

Turner, T.B.: Protective Antibodies in the Serum of Syphilitic Rabbits. J. Exper. Med. 69: 867-889, 1939.

Turner, T.B., Kluth, F.C., McLeod, C., and Winsor, C.P.: Protective Antibodies in the Serum of Syphilitic Patients. Am. J. Hyg. 48: 173-181, 1948.

Touraine, A.: Les Anticorps Syphilitiques; essais de sero-agglutination de la syphilis. Paris: G. Steinheil, 1912.

Eberson, F.: Immunity Studies in Experimental Syphilis; Spirocheticidal Properties of Serums in Latent and Experimental Syphilis with some Observations on Immunity. Arch. Dermat. and Syph. 4: 490, 1921.

Ledbetter, R. K., Jr., Martens, V.E.; The Treponema pallidum Immobilization Test. Bureau of Medicine & Surgery, U.S. Naval Medical School, National Naval Medical Center.

Nelson, R.A., Jr.: Factors Affecting the Survival of Treponema pallidum in vitro. Am. J. Hyg. 48: 120-132, 1948.

Nelson, R.A., Jr., and Steinman, H.G.: Factors Affecting the Survival of Treponema pallidum in vitro. Proc. Soc. Exper. Biol. and Med. 68: 588, 1948.

Nelson, R.A., Jr., and Mayer, M.M.: Immobilization of Treponema Pallidum in vitro by Antibody Produced in Syphilitic Infection. J. Exper. Med. 89: 369-393, 1949.

Nichols, H.J. and Hough, W.H.: Demonstration of Spirochaeta Pallida in the Cerebrospinal Fluid: from a Patient with Nervous Relapse following the Use of Salvarsan. J.A.M.A. 60: 108-110, 1913.

Nelson, R.A., Jr., and Diesendruck, J.A.: Studies on Treponemal Immobilizing Antibodies in Syphilis. I. Techniques of Measurement and Factors Influencing Immobilization. J. Immunol, 66: 667-685, 1951.

Rice, F.A.H. and Nelson, R.A., Jr.: The Isolation from Beef Serum of a Survival Factor for Treponema pallidum. J. Biol. Chem. 191: 35-41, 1951.

Turner, T.B. and Nelson, R.A., Jr.: The Relationship of Treponemal Immobilizing Antibody to Immunity in Syphilis. Transactions of the Association of American Physicians. LXIII: 112-117, Philadelphia, Pa. Dorman, 1950.

Krampitz, L.O. and Werkman, C.H.: The Enzymic Decarboxylation of Oxaloacetate. Biochem. Jour. 35: 595-602, 1941.

Krebs, H.A. and Eggleston, L.V.: Biological Synthesis of Oxaloacetic Acid from Pyruvic Acid and Carbon Dioxide. Biochem. Jour. 34: 1383-1395, 1940.

Magnuson, H.J. and Thompson, F.A., Jr.: Treponemal Immobilization Test of Normal and Syphilitic Serums. J.Ven.Dis.Inform, 30:309-320, 1949.

Kabat, E.A. and Mayer, M.M.: Experimental Immunochemistry. p. 103. First Edition. Springfield, Ill. C.C. Thomas Co., 1948.

Nelson, R.A., Jr., Zheutlin, H.E.C., Diesendruck, J.A., and Austin, P.G.M., Jr.: Studies on Treponemal Immobilizing Antibodies in Syphilis. H. Incidence in Serum and Cerebrospinal Fluid in Human Beings and Absence in "Biologic False Positive" Reactors. Am.J. Syph. 34: 101-121, 1950.

Chorpenning, F.W., Sanders, R.W., and Kent, J.F.: Treponemal Immobilization Test Using Organisms from Frozen Testes. Am.J. Syph. 36: 401-406, 1952.

I. FLUORESCENT TREPONEMAL ANTIBODY (FTA) TEST FOR MICROSLIDE DIAGNOSIS OF SYPHILIS

1. Introduction

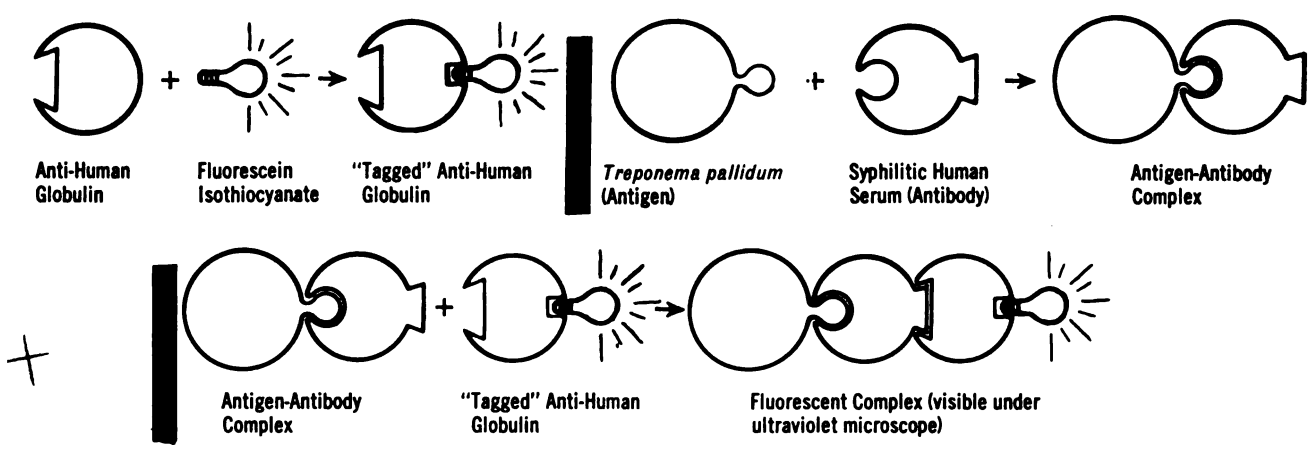
The Fluorescent Treponemal Antibody (FTA) Test is based upon the principles of the fluorescent antibody technique originated and developed by Coons, applied to the microslide diagnosis of syphilis.

Basically, the test is the indirect immunochemical technique for the fluorescent visualization of an antigen-antibody reaction. The reaction is viewed through a microscope equipped with an appropriate light source and filters.

In the FTA test, the patient's serum (antibody) reacts with Treponema pallidum (antigen). Added anti-human globulin, "tagged" with fluorescein isothiocyanate, renders the reaction visible - the treponemes fluoresce with a greenish-yellow light. Although still being evaluated, the FTA test has been shown to produce highly sensitive and specific results.

The following procedure is based on modifications of the FTA test as described in "Serologic Tests for Syphilis," USPHS Publ. #411 (1959):

FLORESCENT ANTIBODY PRINCIPLE: INDIRECT METHOD



2. Materiel:

a. Equipment:

Darkfield fluorescent microscope assembly.
Rotating machine (100 rpm, 3/4" horizontal rotation).
Incubator, adjustable to 37°C.
Water bath, for inactivating sera at 56°C.
Mounting fluid one part buffered saline (pH 7.2), nine parts glycerine (C.P.P).
Plastic microslide box, with cover
Diamond-point pencil.

b. Glassware:
Microslides, frosted end, approximately 1 mm. thickness
Cover glasses (No. 1)
Staining dish with removable glass tray
Serologic test tubes and capillary pipets.

3. Preparations:

a. Reagents:
Treponema pallidum (Nichols Strain) Antigen, desiccated
Anti-Human Globulin, fluorescein-conjugated, desiccated
Phosphate-Buffered Saline, pH 7.2
Phosphate-Buffered Saline, pH 7.2, containing 2.0% Tween-80.

b. Standard Control Sera:

(1) Reactive control serum to demonstrate maximal (4+) or strong reactivity (R), diluted with phosphate-buffered saline as specified for the particular lot of serum.

(2) Reactive control serum to demonstrate minimal (2+) reactivity (R), diluted as specified.

(3) Non-reactive control serum to demonstrate no reaction (N), diluted 1:200 with buffered saline.

4. Controls:

Four control slides accompany each test run of slides on one or more patient sera. Slides A, B and C are prepared with diluted control sera of known reactivity (see "STANDARD SERA" above).

a. Control for maximal fluorescence (R).
b. Control for minimal fluorescence (R).
c. Control for non-fluorescence (N).
d. Control for nonspecific staining; antigen smear treated only with conjugated Anti-Human Globulin.

5. Procedure:

Inactivate all test and control sera, or their dilutions, in water bath at 56°C. for 30 minutes.

a. Blood samples should be collected aseptically, taking care to avoid hemolysis, and allowed to clot. Serum should be separated from the clot, and refrigerated or frozen if not tested immediately. Specimens should be delivered promptly to the laboratory.

b. Restore desiccated T. pallidum antigen by adding 1 ml. of sterile

distilled water to the vial. Mix several times with syringe and needle.

c. Restore desiccated Anti-Human Globulin conjugate by adding 5 ml. of sterile distilled water to the vial. Dispense conjugate in small aliquots and freeze until needed.

NOTE: The restored conjugate must be further diluted, prior to routine use, with phosphate-buffered saline containing 2.0% Tween-80. The dilution factor for each lot of Anti-Human Globulin is determined by testing with Standard Control sera. The dilution selected is optimum when maximal fluorescence is obtained with strongly reactive sera, without using a large excess of diluted globulin. Optimum dilutions have been found in the range 1:5 to 1:320.

d. Prepare a quantity of clean microslides by drawing two 5/8" circles on each, using a diamond-point pencil. Each test, and each control, is run in duplicate on a single slide (see "CONTROLS" above.)

e. Deposit approximately 0.01 ml. T. pallidum suspension, spreading within each circle on all slides. Allow to dry thoroughly, then fix in acetone and air-dry.

f. Add approximately 0.03 ml. of patient serum, diluted 1:200 with phosphate-buffered saline, to a test slide. Test each serum in duplicate on a single slide. Similarly, add the Standard Control Sera, diluted as specified, to control slides A, B and C.

g. Rotate all slides at 100 rpm for 30 minutes with rotator placed in an incubator set at 37°C. Maintain a moist atmosphere, to prevent evaporation, by fixing a piece of moist filter paper in the cover of a plastic slide box. Place slide box in an upright position, insert slides, and cover. Attach box securely to platform to prevent tipping.

h. Rinse slides with buffered saline; soak in two changes of buffered saline for a total of 10 minutes; and drain. Blot slides gently with filter paper.

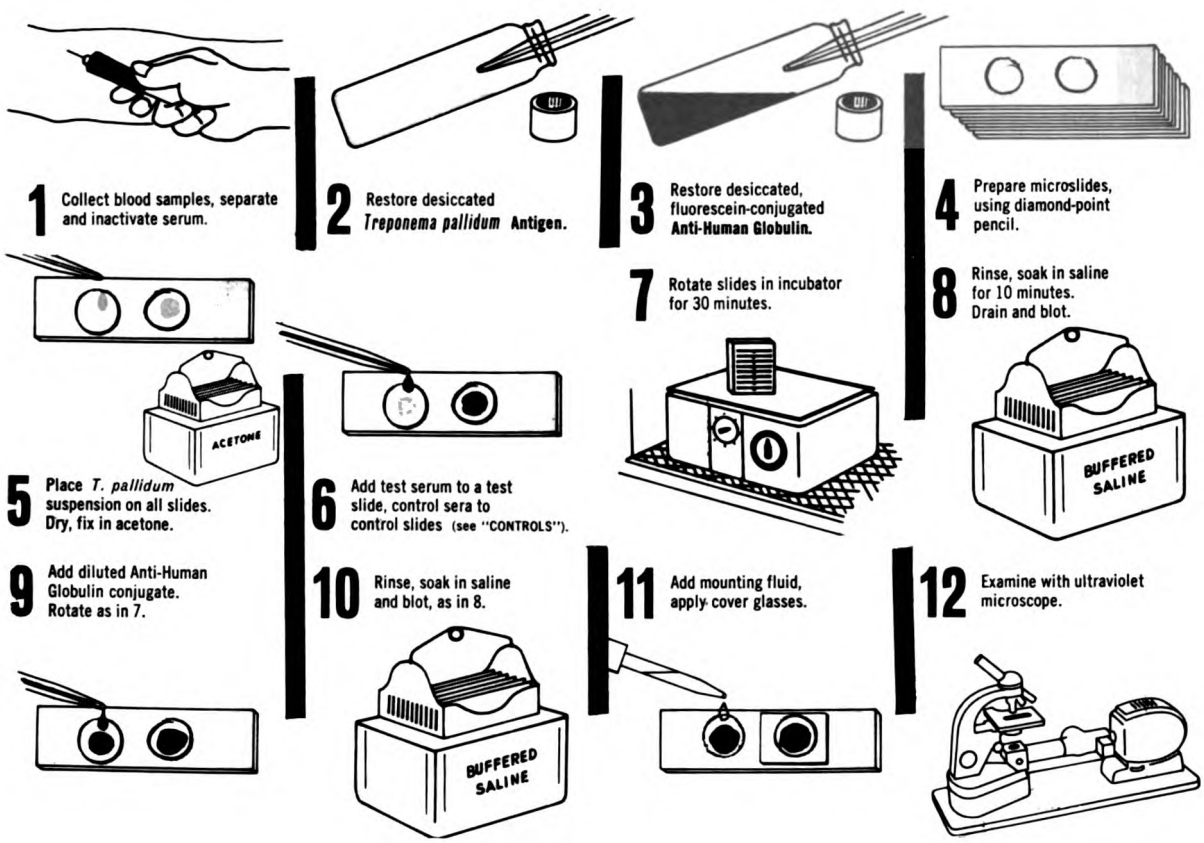
i. Add approximately 0.03 ml. restored and diluted fluorescein-conjugated Anti-Human Globulin to both circles on all slides. Spread conjugate to completely cover the smears. Rotate slides again as in "g" above.

j. Rinse and soak slides again as in "h" above and blot gently with filter paper.

k. Add a very small drop of mounting medium to each smear and supply cover glasses.

l. Examine with fluorescence microscope, using 450X magnification (high-dry objective). Use ordinary darkfield examination to confirm presence of spirochetes where organisms are not fluorescent with ultraviolet illumination (e.g., Nonreactive (Negative) Control).

Table 31. Procedure for FTA Test



REFERENCES

Coons, A.H., Creech, H.J., Jones, R.N. and Berliner, E.: J.Immunol. 45: 159. 1942.

Coons, A.H., and Kaplan, M.H.: J.Exper.Med. 91: 1, 1950.

Deacon, W.E., Flacone, V.H. and Harris, A.: Proc.Soc.Exper.Biol. & Med. 96: 477, 1957.

Deacon, W.E., Freeman, E.M. and Harris, A.: Proc.Soc.Exper.Biol. & Med. 103: 827, 1960.

Cherry, W.B., Goldman, J., Carski, T.R. and Moody, M.D.: Fluorescent Antibody Techniques in the Diagnosis of Communicable Diseases. U.S. Public Health Service, Publication #729, 1960.

J. EVALUATION OF SEROLOGIC TESTS FOR SYPHILIS

1. True Positive Reagin Reactions

Ten days to two weeks after the appearance of the primary lesion 70% to 80% of reagin tests become reactive, reach a maximum of up to 100% reactive during the secondary stage, and fall to 80% to 90% reactive in later stages. The reactivity is altered with antibiotic treatment and subsequently may become completely non-reactive.

2. False Negative Reagin Reactions

Such reactions occur in all stages except possibly the secondary stage of syphilis.

3. False Positive Reagin Reactions

Such false reactions may be due to:

(a) Technical error: It is extremely important that the test procedures be followed explicitly.

(b) Presence of disease other than syphilis: The presence of malaria, leprosy, lupus erythematosus, polyarteritis nodosa, infectious mononucleosis, immunization, and many others.

4. True Positive Reiter Protein and TPI

These become positive later in the first stage and reach their maximum at the end of a year. They remain positive probably for life.

5. False Positive TPI

Related to improper technique.

6. False Negative TPI

The TPI test is less sensitive than the reagin. During early syphilis "positive" reagin and "negative" TPI are noted.

7. FTA - This test parallels the activity of the TPI test.

K. SELECTED BIBLIOGRAPHY FOR SEROLOGY OF SYPHILIS

Beerman, H., Nicholas, L., Schamber, I. L., Greenberg, M. S.: Syphilis: Review of the recent literature. 1960-1961. Arch. Internal Med. (Chicago) 109:323-44, March 1962.

Deacon, W. E., Hunter, E. F.: Treponemal antigen as related to identification and syphilis serology. Proc. Soc. Exp. Biol. Med., 110:352-6, June 1962.

Edwards, E. A.: Detecting Treponema pallidum in primary lesions by the fluorescent antibody technique. Public Health Rep., 77:427-30, May 1962.

Elkerton, L. E.: Serodiagnosis of syphilis - current status. Canad. Med. Ass. J., 86:459-61, 10 March 1962.

Fowler, E., Allen, R. H.: Studies on the production of Wasserman reagin. J. Immunol., 88:591-4, May 1962.

Greaves, A. B.: A comparative study of serologic tests in early syphilis. Arch. Derm. (Chicago), 85:641-3, May 1962.

Harvey, A. M.: Auto-immune disease and the chronic biologic false positive test for syphilis. J. AMA, 182:513-8, 3 November 1962.

Kent, J. F., Covert, S. V., Reilly, H. W., Kench, W. H., Lawson, W. B.: Evaluation of fluorescent treponemal antibody (R FTA and FTA II) and other tests (R PCF and TPI) for syphilis. Proc. Soc. Exp. Biol. Med., 109: 584-9, March 1962.

McLeod, C. P.: Four studies on Treponema pallidum complement fixation antigen. Public Health Rep., 77:431-51, May 1962.

Hunter, C. A. and Burdorff, R.: Serologic tests for typhoid fever. Am. J. Clin. Path., 37:162-7, February 1962.

SECTION IV

CEREBROSPINAL FLUIDS

A. INTRODUCTION

Cerebrospinal fluid is normally clear and colorless; it does not show clot formation on standing. Spinal fluids from healthy persons will show, in the great majority of cases, no cells, some will show 2 to 3 cells per cubic millimeter, and rarely, if ever, will one show more than 5 cells per cubic millimeter. The presence of more than 5 cells per cubic millimeter then can be looked upon as abnormal. All counts between 5 and 10 per cubic millimeter nearly always indicate disease, and counts above 10 per cubic millimeter are practically pathognomonic of disease of the brain or its meninges. Cell counts should be done as soon as possible after collection of the fluid, since the cells rapidly disintegrate, especially in turbid or purulent fluids. The cell count is always done on the second tube of spinal fluid collected which should be free of blood. Fair accuracy may often be had on cell counts ranging between 10 and 200 even after 24 or 48 hours, provided the cells have not been enmeshed in a clot and the fluid has been protected from contamination and kept in a refrigerator. Normal lumbar cerebrospinal fluid protein consisting of albumin and globulin in a ratio of about 5 to 1 varies between 15 mg. and 45 mg. per 100 ml. with an average of around 28 mg. per 100 ml.

Ventricular fluid contains between 5 mg. and 15 mg. per 100 ml., cisterna magna fluid between 15 mg. and 25 mg. per 100 ml, and lumbar fluid between 15 mg. and 45 mg. per 100 ml. The higher values in the lumbar fluid are believed to be a result largely of the relative stagnation occurring in that locality.

1. Record of Physical Characteristics

State whether colorless, yellowish, reddish, greenish, grayish, and so forth, and whether perfectly clear, opalescent, faintly turbid, or purulent; describe the nature of any clot or sediment present.

2. Cell Count

Use the same counting chamber and white blood cell counting pipet as for hematology, and make an accurate count of all red and white cells present. Unna's polychrome methylene blue stain is very satisfactory for use in distinguishing between white and red cells and in differentiating the types of white cells when their number is not great. It is prepared by dissolving 1 gm. of methylene blue and 1 gm. of potassium carbonate in 100 ml. of distilled water

and ripening for immediate use if desired by heating at 60°C. for 10 minutes. The addition of a crystal of thymol will prevent the growth of molds. This stock solution should be kept in the refrigerator and filtered before using, when necessary. To insure an even distribution of any cells present, the fluid should be shaken thoroughly. Decant a small sample of the fluid into a separate, clean, dry tube to avoid contaminating the main specimen with stain. The dye is drawn up to the 0.5 mark in the pipet used for counting white blood cells and the fluid is then drawn up to the 11 mark. This dilution produces a slight error which is negligible for clinical purposes. The fluid and the stain are shaken in the pipet for 1 minute. The counting chamber of 0.1 mm. depth, ordinarily used for counting of blood, is satisfactory for use with cerebrospinal fluid. When the fluid is stained as outlined, the nuclei of the white cells appear light blue and the cytoplasm is colorless and only faintly visible. The red blood cells appear slightly yellow. The whole ruled space of 9 square millimeters is counted, unless the cell count is so high that counting a smaller area will give an average value, which may be multiplied to give the count in the entire field. The total number of cells is multiplied by 10/9 to obtain the number per cubic millimeter, as the field is 9 square millimeters in area. For still greater accuracy and to overcome the slight error mentioned, this result can then be multiplied by 20/19 to correct for the dilution with stain.

a. Cerebrospinal fluid containing visible amounts of blood is not suitable for total cell counts because the leukocytes present result in counts that are too high. Even traces of blood may result in diagnostic errors in diseases in which the increase of leukocytes is between 20 and 100 cells per cubic millimeter.

b. When a more accurate differential determination of the white cells present is desired than is afforded by Unna's stain, a portion of the fluid is centrifuged at a moderate speed for several minutes, the supernatant fluid is decanted, and a drop of the sediment is smeared on a slide, allowed to dry, and stained with Wright's stain.

B. TESTS FOR INCREASED GLOBULIN

1. Ross-Jones Test

This test is performed with a saturated solution of ammonium sulfate. A supersaturated solution is made by dissolving about 80 Gm. of ammonium sulfate in 100 cc. of hot water. Some crystals will separate out on cooling; the solution is then known to be saturated. In performing this test in connection with the preparation of concentrated globulin solution, 1.5 ml. of the saturated ammonium sulfate is allowed to run in from a pipet held close to the bottom of the standard serologic tube containing the 1.5 ml. of spinal fluid. The solution is underlaid carefully so that there will be no mixing. If globulin is present, a turbid white ring appears at the junction of the fluids within 2 minutes. If it is

not seen within 2 minutes, the test is negative. (Globulin is precipitated by a half-saturated ammonium sulfate solution. The point of half saturation is at the junction of the saturated solution of ammonium sulfate and the cerebrospinal fluid.) The test should not be read after 2 minutes.

2. Pandy's Test. To 1 cc. of a saturated aqueous solution of phenol in a small test tube, add 1 drop of spinal fluid. A bluish-white ring is immediately formed if an excess of globulin is present. This test is more sensitive than the Ross-Jones test.

3. Colloidal Gold Test

Lange's colloidal gold test is performed by mixing cerebrospinal fluid in varying proportions with a colloidal suspension of gold chloride. Reactions are indicated by a color change. The test should not be conducted on cerebrospinal fluid containing blood because false positive and variable reactions may be produced. Normal spinal fluids produce no precipitation of the gold solution and the color remains orange pink. Complete precipitation produces a colorless solution and intervening degrees of precipitation range through a color change of red, lilac and blue. Degrees of precipitation or color change are indicated by numbers as follows:

5 - Colorless, complete precipitation
4 - Pale blue
3 - Blue
2 - Purple or lilac
1 - Red or bluish red
0 - Orange pink, no precipitation

Many pathologic conditions of the central nervous system produce no change. Many cases of syphilis of the central nervous system and inflammatory conditions of the meninges produce marked changes, but they are of diagnostic importance only insofar as they indicate abnormality of the protein content of the fluid. The terms "paretic," "syphilitic," and "meningitic" curves which formerly were used to describe these changes have been discarded for the terms "first zone," "mid-zone," and "end zone" reactions.

a. Reagents and materials

(1) Colloidal Gold Solution:

Standard colloidal gold solution is prepared for naval medical activities at the United States Naval Medical School and may be obtained upon written request.

Each colloidal gold solution has its own specific acid solution and this cannot be interchanged with that of another lot. For preparing this solution, sufficient alkaline colloidal gold for immediate use is removed from the stock container. To each 30 ml. (amount sufficient for one rack), add the indicated

amount of the accompanying standard acid solution. Acid should only be added just prior to use. Do not add acid to the bottle of stock solution.

(2) Saline Solution:

The solution used is an accurately prepared 0.4% sodium chloride solution. This should be prepared by dissolving 4.0 Gm. of anhydrous reagent grade sodium chloride (accurately weighed) in redistilled water and diluting to a volume of 1,000 ml. in a volumetric flask.

(3) Test Tubes:

The tubes used are 15 by 100 mm. in size. Special precautions to insure chemical cleanliness are necessary. The test tubes should be washed with soap and water, rinsed in tap and distilled water and dried, following which they are placed in aqua regia. (This is one part of nitric acid and three parts of hydrochloric acid. This potent mixture should be kept under a hood with the additional precaution to keep the lid slightly ajar when the mixture is first made as the potent fumes may blow off the glass cover. Make the mixture in a glass container). See SECTION II-C - PREPARATION OF GLASSWARE for further steps in cleaning these tubes (p.13).

(4) Pipets

Ten ml. pipets graduated to 0.1 ml. and 1 ml. pipets graduated to 0.01 ml. are used.

b. Procedure

(1) Place 11 clean, dry test tubes in a row in a rack.

(2) Add 1.8 ml. of saline solution (0.4% sodium chloride) to the first tube and 1.0 ml. to each of the other 10 test tubes.

(3) Add 0.2 ml. of cerebrospinal fluid to the first tube and mix thoroughly. This makes a 1 to 10 dilution. Transfer 1.0 ml. from the first tube to the second tube, making a 1 to 20 dilution. The process is continued down through the tenth tube from which 1.0 ml is discarded. The dilutions are 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120. The eleventh tube is used as a control.

(4) Add 5 ml. of gold solution to each tube. Shake well, cover, place in dark, and let stand for 24 hours at room temperature.

c. Modified Colloidal Gold Test

For economy, the procedure may be carried out with one-half the amounts mentioned.

(1) Place 11 chemically clean test tubes in a rack.

(2) Into the first tube place 0.9 ml. of 0.4% sodium chloride solution and 0.5 ml. in each of the remaining 10 tubes.

(3) Add 0.1 ml. of spinal fluid to the first tube and thoroughly mix.

(4) Transfer 0.5 ml. to tube 2; mix thoroughly and remove 0.5 ml. and place in tube 3; continue until the tenth tube is reached and then discard 0.5 ml. from this tube. The eleventh tube is used as a control.

(5) Add to each tube 2.5 ml. of colloidal gold solution.

(6) Shake well, cover, place in dark and let stand for 24 hours at room temperature. The readings are now made and recorded in the same way as in the original test.

Gold curve---Tube---	1	2	3	4	5	6	7	8	9	10
5 colorless										
4 pale blue										
3 blue										
2 purple or lilac										
1 red or bluish red										
0 orange pink										

(7) The test is reported by the number of the color reaction for each of the ten tubes, e.g., 4443210000.

d. Reading Technique

The test is read at 24 hours. The rack with tubes is placed in front of a white background with good ordinary lighting (same type of light should be used for each test). The tubes are then graded, comparing them to the control tube. It should be noted that recognition of the color changes depends on acquiring experience in reading the tests, and such experience should be relied upon rather than depending on color comparison charts.

e. Interpretation

Any color change less than "lilac" may be considered of no significance. Any color change greater than "lilac" should be considered as definite evidence of an abnormal fluid. Color changes to and including "lilac" are seen in many normal fluids.

(1) Normal Curve:

The following is an example of a normal curve: 0011000000. It is noted that the normal curves may include such reports as 0022110000. The presence of these curves is due to more sensitive reagents, the presence of novocaine in the spinal fluid, and/or the presence of blood.

(2) First Zone Curves:

These are the reactions showing partial or complete precipitation in the first few test tubes. They occur so frequently in spinal fluids of patients with syphilis of the central nervous system and multiple sclerosis that they are

almost of diagnostic importance. They are seen in about 80% of patients with paresis, 40% with syphilitic meningitis, 8% with tabes dorsalis, 15% with cerebrovascular neurosyphilis, 22% with syphilis of the spinal cord, 9% with asymptomatic neurosyphilis and in practically all cases of syphilitic optic atrophy. The following is an example of a typical first zone curve: 5554432100.

(3) Mid-Zone Curves:

In these reactions the greatest change is noted in the fourth to sixth tubes. They rarely are of a degree to produce complete precipitation. They may be found in any abnormal fluid, but their presence is of no differential diagnostic significance. An example of this curve is 0012332100.

(4) End Zone Curves:

The greatest changes are to be seen in the 6th to the 10th test tubes, but they rarely show complete precipitation. This type of curve is seen in spinal fluids with a very high protein content and especially when the albumin-globulin ratio is high. Such conditions are acute purulent meningitis, subarachnoid block, and hemorrhage into the subarachnoid space. This curve is reported 0000112344.

SECTION V

ANTISTREPTOLYSIN O TITRATIONS

A. INTRODUCTION

The purpose of this procedure is to determine whether the serum of a patient contains a significant level of antibody for one of the hemolysins elaborated by beta-hemolytic Streptococci; thus, the hemolysin is called Streptolysin O and its antibody is known as Antistreptolysin O(ASO). A significant rise in ASO titer, during the course of an illness, provides an aid in the clinical diagnosis of rheumatic fever and acute glomerulonephritis; an ASO titer in excess of 166 Todd units, in a single determination, is considered abnormally high and suggests that the patient has had a recent infection with Streptolysin O-producing streptococci. A Todd unit which is an expression of the antibody (ASO) content of a serum, is the reciprocal of the highest dilution of serum (that is, the smallest amount of serum in dilution) that completely inhibits hemolysis in the test. The materials required, details of the test, and interpretation of results are given below.

B. MATERIALS

All glassware should be chemically clean; pipets, test tubes, and other containers must be free of lipids, detergents, and other extraneous substances. Serum and reagents employed in the test are:

1. Patient's Serum

Patient's serum, which must be clear, non-hemolyzed, and uncontaminated. The amount provided is preferably from 0.6 to 1.0 ml.; it is desirable to use 0.5 ml. for each test, whereas the minimum volume required is 0.3 ml.

2. Streptolysin O Buffer Solution

Completely dissolve 12.4 Gm. of dehydrated Bacto-Streptolysin O Buffer (Difco) in 1,000 ml. of distilled water; the solution is stored in a screw-capped bottle in the refrigerator at 2^{o} to 6^{o} C. This buffer solution is used in preparing dilutions of the patient's serum and of the known standard serum and for making the red cell suspension.

3. Streptolysin O Reagent

To the vial of Bacto-Streptolysin O Reagent (Difco), add the amount of cold, freshly distilled water specified on the vial (e.g., 25 ml.), then invert the vial

several times until the material is completely dissolved. The reagent should not be rehydrated and dissolved more than 10 minutes prior to its use in the test.

4. Red Cell Suspension

Erythrocytes may be obtained from freshly drawn whole human or rabbit blood or from a blood bank; it is preferable to use blood type O red cells. The red cells are washed in physiologic saline solution 3 times or until the supernatant is perfectly clear and colorless; if more than 5 washings are required, the cells are not suitable for use. Cells are washed by centrifuging the blood, siphoning off the supernatant, resuspending the cells in saline solution, centrifuging at 2000 rpm for 3 to 5 minutes, and repeating this process until the supernatant is colorless (except as noted above). The red cell suspension for the test is made by mixing 5 ml. of the packed cells with 96 ml. of buffer solution (see 2 above).

5. Known Standard Serum

This is used as a control of the reagents in the test and, under proper conditions, should indicate a well-defined end-point in the middle tube of the dilution series used. To the vial of dehydrated Bacto-ASTO Standard (Difco) aseptically add 10 ml. of sterile distilled water, shake the vial gently to effect complete solution, but avoid formation of foam or bubbles; this solution represents a 1:100 dilution of the original serum and is used as shown below. Required amounts of the rehydrated standard should be removed aseptically and the balance stored in the refrigerator at 2° to 6° C.

C. TEST PROCEDURES

1. Preliminary Dilutions of Patient's Serum

Label each of 3 test tubes (16 x 150 mm.) with the specimen number and the letters A, B, and C respectively; using a 10 ml. pipet, add buffer solution to the tubes—4.5 ml. to tube A, 9 ml. to tube B, and 8 ml. to tube C. Next, with a 1 ml. pipet add 0.5 ml. of the patient's serum to tube A and mix well by alternately drawing up the fluid into the pipet and forcing it out gently at least 10 times, avoiding formation of air bubbles or foam; leave the pipet in the tube. Useing another 1 ml. pipet, transfer 1 ml. of dilution A to tube B and mix the contents as described for dilution A and leave the pipet in the tube. Then, with a 5 ml. pipet, transfer 2 ml. of dilution B to tube C and mix as described above; discard the 5 ml. pipet and replace it with a 1 ml. pipet in tube C. The 3 tubes now contain diluted patient's serum as follows:

Tube A	1:10 dilution (0.5 ml. serum + 4.5 buffer solution)
Tube B	1:100 dilution (1.0 ml. dilution A + 9 ml. buffer solution)
Tube C	1:500 dilution (2 ml. dilution B + 8 ml. buffer solution)

NOTE: Retain these 3 tubes of diluted serum until results of the test have been read and found satisfactory.

2. Titration Dilutions of Patient's Serum

Place 12 test tubes (13 x 100 mm.) in a continuous row in a rack and label each one as described in the next sentence. On Standard Form 514, showing a request for ASO titer of the patient's serum, write a number to be used for identifying the series of tubes containing dilutions of that patient's serum; thus, the tubes for serum #1 in the first row would be labeled 1-1, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, and 1-10. Label the other 2 tubes #13-RC (for the red cell control), and #14-StC (for the streptolysin control), respectively; tubes numbered 11 and 12 are omitted at this point.

a. Since it is economically desirable to test as many patient's serums as possible with each vial of streptolysin O reagent, each rack should contain the appropriate number of series of 10 tubes for each serum; only one set of controls is necessary for each vial of reagent. Thus, the number of 25 ml. vials of reagent required for titrating different numbers of serums are: 1 vial - 4 serums; 2 vials - 9 serums; 3 vials - 14 serums; 4 vials - 19 serums; and, 5 vials - 24 serums.

b. The next step is to add buffer solution to each series of tubes, using a 1 ml. pipet for this purpose; amounts of buffer solution per tube in each series are as follows:

Tube 1 - 0.2 ml.	Tube 5 - 0.4 ml.	Tube 9 - 0.2 ml.
" 2 - 0.8 "	" 6 - 0.6 "	" 10 - 0.4 "
" 3 - none	" 7 - 0.7 "	" 13 - 1.5 "
" 4 - 0.2 ml.	" 8 - none	" 14 - 1.0 "

Using the 1 ml. pipets that were left in the tubes of preliminary serum dilutions, add amounts of diluted serum to the titration series of tubes as given below; resulting tube dilutions of serum are shown in the right hand column.

From tube	A	- add	0.8	ml.	to tube	1	=	1:12 1/2
"	"	"	0.2	"	"	2	=	1:50
"	"	"	1.0	"	"	3	=	1:100
"	"	"	0.8	"	"	4	=	1:125
"	"	"	0.6	"	"	5	=	1:166
"	"	"	0.4	"	"	6	=	1:250
"	"	"	0.3	"	"	7	=	1:333
"	"	"	1.0	"	"	8	=	1:500
"	"	"	0.8	"	"	9	=	1:625
"	"	"	0.6	"	"	10	=	1:833

3. Titration Dilutions of Known Standard Serum

A series of 5 tube dilutions of this positive standard should be included in each test series of patients' serums. Place 5 test tubes (13 x 100 mm.) in the rack (preferably in the row containing the red cell and streptolysin control tubes); label these tubes PS-3, PS-4, PS-5, PS-6, and PS-7, respectively. Using a 1 ml. pipet, add buffer solution to these tubes; then, with another 1 ml. pipet, add rehydrated positive standard serum. The amounts of each and the resulting dilutions of positive standard serum (PS) are given below.

Tube	PS Solution	Buffer Solution	Serum Dilution
PS-3	1.0 ml.	none	1:100
PS-4	0.8 "	0.2 ml.	1:125
PS-5	0.6 "	0.4 "	1:166
PS-6	0.4 "	0.6 "	1:250
PS-7	0.3 "	0.7 "	1:333

Gently shake the rack containing the tubes of diluted patients' serums, known standard serum, and the control tubes, to mix the contents.

4. Streptolysin O Reagent Solution

Using a 5 ml. pipet, add 0.5 ml. of the reagent solution to each tube in the rack EXCEPT that labeled 13-RC (red cell control). Shake the rack of tubes gently to mix the contents, then place it in the incubator at 37° C. for 15 minutes.

5. Red Cell Suspension

Remove the rack of tubes from the incubator, then, using a 5 ml. pipet, add 0.5 ml. of the red cell suspension to each tube, including both controls. Gently shake the rack of tubes to mix the contents, then place it in the incubator at 37° C. for 15 minutes, after which the rack is shaken gently again and reincubated for 30 minutes at 37°C. At the end of the 45 minute incubation period, remove the rack of tubes, check the labels if necessary, then centrifuge the tubes at 1000 rpm for 1 minute.

6. Reading and Interpretation of Results

Replace the tubes in the rack in their proper order, then examine the two controls (tubes 13-RC and 14-StC). Tube 14-StC should show marked to complete hemolysis as indicated by the presence of a pink or cherry-red color in the supernatant fluid and the absence of intact red cells in the bottom of the tube; tube 13-RC should present no evidence of hemolysis. Next, examine the 5 tubes containing dilutions of known positive standard serum; tubes PS-6 and PS-7 should

show hemolysis, whereas tubes PS-3, PS-4, and PS-5 should show no hemolysis, thus indicating a content of 166 Todd units in the serum. Finally, examine the 10 tubes containing dilutions of patient's serum to determine the end-point in the titration which is the highest dilution in which no hemolysis has occurred. Thus, if there is no hemolysis in the first 8 tubes, but marked hemolysis in tubes 9 and 10, the end-point dilution is 1:500 and, by definition, the patients' serum contained 500 Todd units and is so reported.

7. Additional Dilutions of Patients' Serum

Serum from certain patients may contain more than 833 Todd units; that is, no hemolysis will occur in any of the 10 dilutions routinely used. In such circumstances, it may be desirable to test 2 higher dilutions of the patients' serum as a supplement to the first titration; the two extra tube dilutions are made from the preliminary dilution in tube C - 1:500. Label the tubes with the identifying serum number and tube number, for example 1-11 and 1-12. Using the appropriate 1 ml. pipet, first add buffer solution, then diluted serum from tube C in the amounts shown below; the serum dilutions in tubes 11 and 12 are also given.

	Buffer solution		Tube C serum dilution		Tube dilution
Tube 11	0.6 ml.	+	0.4 ml.	=	1:1250
"	0.8 "	+	0.2 "	=	1:2500

Shake the tubes gently to mix the contents, then proceed as described above for the first titration. If hemolysis occurs in tubes 11 and 12, but none appeared in the first 10 tubes, the patients' serum contained 833 Todd units; if there is no hemolysis in tube 11, but is marked in tube 12, the ASO content of the patient's serum was 1250 Todd units. If hemolysis occurs in neither tube 11 nor tube 12, the patient's serum contained more than 2500 Todd units and is so reported.

8. Summarization of ASO Titration Procedure

A tabular summarization of the procedure for ASO titration is given on the following page.

ANTISTREPTOLYSIN O TITRATIONS

Materials in tubes	Preliminary serum dilutions												Controls	
	A 1:10		B 1:100					C 1:500					RC	StC
Dilution tube number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Amt. patient's serum -ml.	0.8	0.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2	0	0
Strep. O buffer sol. -ml.	0.2	0.8	0	0.2	0.4	0.6	0.7	0	0.2	0.4	0.6	0.8	1.5	1.0
(Shake rack gently to mix tube contents)														
Strep. O reagent sol. -ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5
(Shake rack gently to mix tube contents; incubate @ 37° C for 15 min.)														
5% red cell susp. -ml.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pos. stand. serum-ml.			1.0	0.8	0.6	0.4	0.3	* (See footnote)						
Strep. O buffer sol. -ml.			0	0.2	0.4	0.6	0.7							
Strep. O reagent sol. -ml.			0.5	0.5	0.5	0.5	0.5							
5% red cell susp. -ml.			0.5	0.5	0.5	0.5	0.5							
(Shake rack gently to mix tube contents; incubate at 37° C for 15 minutes, shake rack gently again, then reincubate at 37° C for 30 minutes. Centrifuge all tubes for 1 minute at 1000-1500 rpm, then replace in rack and read results)														
Todd units represented	12	50	100	125	166	250	333	500	625	833	1250	2500	0	0

*NOTE: These preparations are made in tubes placed in the rack containing patients' serums and thus are subjected to the same procedures.

D. INTERPRETATION

The presence of ASO titers in excess of 1:166 are abnormally high. The exact point at which one considers the titers to be elevated depends on the individual laboratory. The ASO titer indicates only the presence of previous streptococcal infection, particularly when associated with a rising titer (repeat determination) or persistently high levels in excess of 1:333.

E. REFERENCE

Todd, E.W.: Antihemolysin titers in hemolytic streptococcal infections and their significance in rheumatic fever. Brit.J.Exper.Path. 12:248-259, 1932.

SECTION VI

WEIL-FELIX REACTION AND WIDAL TEST

A. INTRODUCTION

These are the "Febrile Agglutinations" and are based upon the fact that patients ill with certain fever-producing (febrile) infections respond by the development of antibodies which, by using serum from the patient, will cause agglutination (clumping) of bacteria in standard suspensions prepared from pure cultures. Satisfactory preparations of several different bacterial suspensions (bacterial antigens) are available commercially (Lederle Laboratories and Markham Laboratories). Antigens for use in Widal tests include S. typhi O (somatic) and H (flagellar), S. paratyphi A O and H, S. paratyphi B O and H, S. paratyphi C O and H, and B. abortus; those employed in Weil-Felix reactions consist of Proteus OX-19, Proteus OX-K, and Proteus OX-2. Although strains of the bacteria Proteus vulgaris and rickettsial organisms are not closely related, they share certain antigens in common. Often persons suffering from rickettsial diseases will develop antibodies that act not only against the infecting organism but also against Proteus vulgaris. This strange phenomenon is the basis of the Weil-Felix reaction. For example, if dilute serum produces agglutination in a suspension of Proteus OX-19 bacteria, this suggests the patient has epidemic typhus. A similar reaction with Proteus OX-2, in addition, indicates Rocky Mountain Spotted fever. Agglutination of the strain OX-K points to scrub typhus, while a negative result to all three antigens is found in Q-fever.

The Widal Test is a more specific serologic technique for detecting elevated antibodies. It is used in the diagnosis of the typhoid-paratyphoid group of diseases. The causative organisms belong to the Salmonella family. Each strain of these bacteria has both somatic ("O" type) and flagellar ("H" type) antigens, which produce corresponding antibodies in the infected patient. The antibody (agglutinin) is quantitated by a serial tube dilution method similar to that employed in the Weil-Felix reaction. The end-point in both procedures is the "weakest" dilution of serum that can cause significant clumping of the bacterial suspensions. The test includes also the agglutination test for Brucella abortus. Its reaction is similar to that of the Salmonella group.

B. MATERIALS

1. Patient's Serum

Patient's serum must be clear, non-hemolyzed, and uncontaminated. The amount required for testing with 12 different antigens is a minimum of 2 ml.; it is desirable, therefore, to collect serum obtainable from approximately 10 ml. of whole blood.

2. Bacterial Antigens

These are commercially prepared bacterial suspension of the species listed above; the antigens are available in 5 ml. vials with attached standardized droppers. The delivery end of the dropper is curved to facilitate accurate dispensing; a single drop of the antigen is approximately 0.03 ml.

3. Glassware

Glassware required consists of 1 serologic pipet (Kahn), 0.2 ml. graduated in thousands, for each serum to be tested, and a glass slide, or slides, on which the tests are made. A satisfactory slide can be prepared from a piece of clear, scratch-free picture frame or window glass measuring about 9 x 14 inches; using a diamond-point pencil, a series of 1-1 1/2 inch ruled squares are marked off in rows of 5 or 6 across the shorter width of the slide, thus providing at least 12 rows across the longer portion of the slide. If preferred, Boerner microtest slides may be used; each of these slides is constructed with 2 rows of 5 wells so that 6 slides would be required to test a single serum in 5 dilutions against 12 bacterial antigens. After use, slides should be rinsed with hot water, cleaned with Bon Ami (or similar material), then dried and polished with a clean, smooth cloth. Slides should be polished again just prior to use.

4. Applicator Sticks

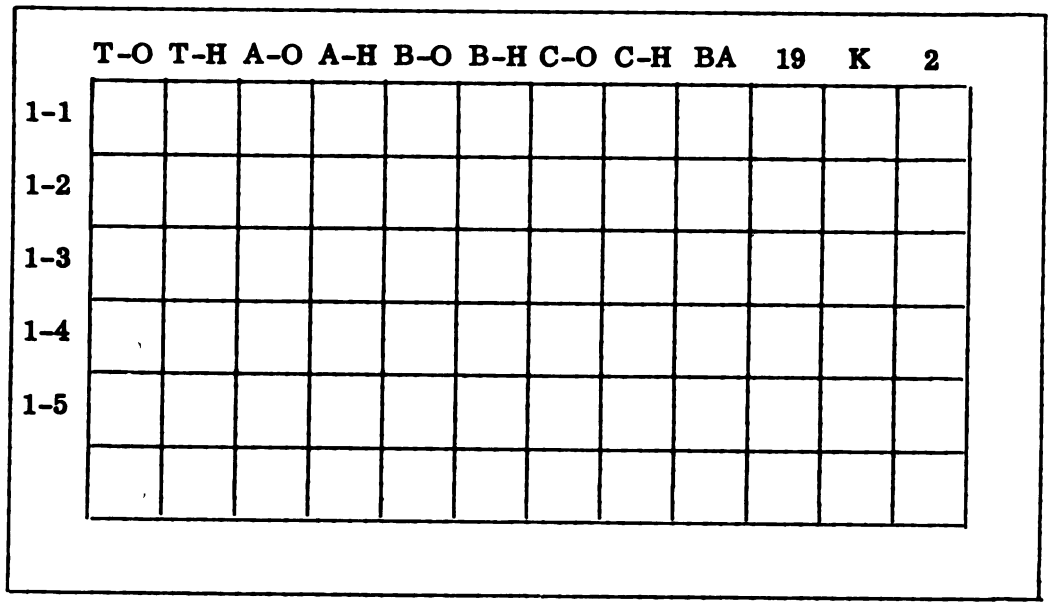
These are plain, wooden applicator sticks, 6 1/4 inches long and 1/12 inch in diameter. They are used for mixing serum and antigen preparations on the glass slides.

C. TEST PROCEDURES

1. Method

Label the glass slide (or slides) with numbers or letters to identify the patient's serum and the antigen used in each series of serum dilutions. Then, using a 0.2 ml. pipet, place the following amounts of serum in the center of each of 5 squares (or wells) in the first row, moving from top to bottom: 1st - 0.08 ml.; 2nd - 0.4 ml.; 3rd - 0.2 ml.; 4th - 0.01 ml.; and 5th - 0.005 ml. This procedure is repeated for each successive row of 5 squares until 12 rows of 5 squares each contain appropriate amounts of serum (see Fig. 3 on following page.)

Fig. 3 . Glass plate ruled into squares for use in febrile agglutination tests.



a. Shake the vial of S. typhi O antigen to obtain an even suspension, remove and hold the dropper nearly parallel to the slide with the delivery end about 1/2 inch above the slide and allow 1 drop of antigen to fall free onto each of the 5 squares in the first row (labeled T-O in Fig.3). Care must be exercised to avoid touching the slide or drops of serum with the tip of the dropper. Replace the dropper in the vial. Then, using a clean applicator stick, thoroughly mix serum and antigen in each square, starting with the one containing the smallest amount of serum (square #5) and moving from bottom to top (1-5, 1-4, 1-3, 1-2, and 1-1 respectively in Fig. 3).

b. Repeat this process, using S. typhi H antigen for row T-H, S. paratyphi A O antigen for row A-O, etc. (Fig. 3), until the appropriate antigens have been added and mixed with serum in all 60 squares; a new, clean applicator stick must be used for mixing serum and antigen in each row of squares. Next, the slide is held in a manner such that lighting adequate for clear visibility of agglutination is provided, and the slide is slowly rotated and alternately tilted back and forth for 3 minutes or until definite agglutination occurs. Caution must be observed to prevent any of the mixtures flowing from one square to another.

NOTE: The procedure just described must be conducted quickly to avoid drying of fluids in the first rows before materials in the last row are mixed, because flakes of dried mixture may be mistaken for partial agglutination; if too much time is consumed in meeting these requirements, the technician should divide the test into 2 or 3 parts on the large slide, or use Boerner slides, thus enabling him to read results under proper conditions.

2. Reading and Recording Results

Hold the slide in a manner such that non-glaring light (either daylight or artificial light from a Dazor lamp with the shield slightly tilted upward) passes upward through the slide to provide maximum visibility of any agglutination that

has occurred. As mentioned above a 2+ (50%) agglutination, or higher, is considered a positive result. Examine closely the contents of each square on the slide, following the order in which the materials were mixed. Note and record the highest dilution of serum that shows a positive test with each antigen. This dilution is the agglutination titer of the serum; absence of any agglutination, or partial agglutination less than 2+, are recorded as negative (see, however, discussion of degrees of agglutination in Weil-Felix reactions below). For purposes of recording and reporting results in terms of serum titers, the values shown below are employed.

Serum - ml.		Antigen - ml.		Equivalent dilution
0.08	+	0.03	=	1:20
0.04	+	0.03	=	1:40
0.02	+	0.03	=	1:80
0.01	+	0.03	=	1:160
0.005	+	0.03	=	1:320
0.002*	+	0.03	=	1:640

*0.02 ml. of a 1:10 dilution of whole serum; used only when necessary to obtain an end-point.

The "equivalent dilutions" shown above are not actual dilutions of serum after mixture with antigen. Instead, the mixtures of antigens with varying amounts of serum shown will test the agglutinating powers of serum that closely approximate the results that would be expected if the serums had been used in the tube agglutination test with the serial dilutions indicated above.

D. INTERPRETATION

A demonstrated rise in agglutinin titer during the course of illness has far greater significance than does a single positive result; thus, a fourfold increase would be considered diagnostically important. A Widal test is considered positive when 50% or higher degree of agglutination occurs in a mixture of antigen and serum dilution; the degrees of agglutination are estimated and recorded as 4+ (complete, 100%), 3+ (75%), 2+ (50%), 1+ (25%), ± (trace to 25%), or - (negative, no agglutination). For example, a serum from a patient might cause 4+ agglutination in equivalent dilutions of 1:20, 1:40, and 1:80; a 2+ agglutination in 1:160 dilution; and no agglutination in 1:320 dilution. The highest serum dilution giving a positive result is, therefore, 1:160 and the titer of the serum is so reported. In the Weil-Felix reactions, the degrees of agglutination may be read simply as negative (no agglutination), complete (100%), or partial (any degree of definite agglutination); a partial agglutination may be considered a positive result, but a minimum of 2+ agglutination is more definitive. The presence of two antigens in the Salmonella group are of additional help in interpreting the results. Thus, a high titer 1:160 or more of "O" agglutinins and a low titer of "H" agglutinins suggest an active infection. A titer of 1:80 or 1:160 of "H" agglutinins is more indicative of past infection or prior immunization.

F ENCES: See General References at end of Section I, page 2 .

SECTION VII

LUPUS ERYTHEMATOSUS TEST
or
LE TEST OF HYLAND LABORATORIES

A. INTRODUCTION

Systemic lupus erythematosus is a disease of protean manifestation involving skin, mucous membranes, serous surfaces, blood constituents, joints, kidneys, heart, lungs, and brain. The disease is associated with various immunological abnormalities and occurs most frequently in young women. The diagnosis is made on the evaluation of a large and complex group of symptoms, signs, and laboratory findings. The most valuable immunologically dependant laboratory test for the disease is the "LE Cell" Test, the technique of which is discussed in the U.S. Naval Medical School Hematology Manual. The "LE" Slide Test described here is less sensitive than the "LE Cell" Test.

B. MATERIALS

The materials for the test are obtained in kit form from the Hyland Laboratories, Los Angeles, California, and consist of the following:

1. Dropper bottle of stabilized reagent (polystyrene latex particles sensitized by exposure to calf thymus nucleoprotein extract).

2. Positive and Negative Control Sera (should be refrigerated when not in use).

3. Capillary tubes.

4. Special slide with an outlined 20 x 40 mm. oval.

C. PROCEDURE

1. Resuspend the Latex-Nucleoprotein Reagent thoroughly by gentle shaking before use.

2. Using the capillary pipets supplied, place a drop of the serum under test in one of the ovals of the slide.

3. Add a drop of Latex-Nucleoprotein Reagent. With a wooden applicator mix and spread the reaction mixture completely over the area within the oval.

4. From time to time, prepare positive and negative controls, each with 1 drop of appropriate LE Test control serum and 1 drop of Latex-Nucleoprotein reagent.

5. Tilt the slide slowly from side to side, and observe for macroscopic clumping. With a positive reaction, flocculation will be seen within 2 minutes.

D. REPORTING RESULTS

The test is reported as "Positive" or "Negative." A positive test is very strongly suggestive of systemic lupus erythematous, but a negative result does not rule out the diagnosis.

E. REFERENCES

Harrison, T.R.: Principles of Internal Medicine. McGraw-Hill, 1962, p. 1892.

Cecil, R.L. and Loeb, Robert F.: A Textbook of Medicine, Vol. I, W.B. Saunders Co., 1959, p. 460.

Dubois, E.L., Drexler, E. and Arterberry, J.D.: A latex nucleoprotein test for diagnosis of systemic lupus erythematosus. A comparative evaluation. JAMA 177:141-3, 15 July 1961.

SECTION VIII

THYROGLOBULIN AUTOPRECIPITIN TEST (TA TEST)

A. INTRODUCTION

Hashimoto's thyroiditis is a disease of the thyroid gland manifested by moderate enlargement (goiter) and a relatively specific pathologic histologic alteration. In 1956 antibodies against components of thyroid gland were demonstrated in the serum of patients with this disease and since that time the disease has been called "Auto-immune Thyroiditis" by many workers.

Several methods for demonstrating thyroid auto-antibodies have been devised. The Hyland TA Test is a relatively simple screening test.

B. MATERIALS

The materials are available in kit form from the Hyland Laboratories, Los Angeles, California.

C. PROCEDURE

1. Inactivate serum specimen at 56°C. for 30 minutes.

2. Prepare an approximately 1:20 dilution of serum by adding one drop of serum to 1 ml. of gylcine-saline buffer diluent.

3. Place one drop of positive control serum (which has already been inactivated and need not be reheated), one drop of the test serum and one drop of the diluted test serum into separate sections of the divided glass slide.

4. Add one drop of Latex-Thyroglobulin reagent to each serum. Using separate applicators or toothpicks, mix and spread each reaction mixture over an area of approximately 20 x 25 mm.

5. Tilt slide slowly from side to side for 2 or 3 minutes and observe for macroscopic clumping.

6. The reagent, diluent, and control serum should be kept at 2° to 10°C.

D. REPORTING RESULTS

1. It appears necessary to test each serum specimen undiluted and at a dilution of about 1:20. since occasional serums show a prozone which is eliminated

by dilution. A weak specimen may react well undiluted, but may give a "Negative" reaction at the 1:20 dilution; such a specimen should be considered "Positive".

2. The test provides a rapid screening procedure for auto-immune thyroiditis, but false positives may occur in patients with rheumatoid arthritis.

E. REFERENCE

Buchanan, W. Watson: Auto-Immune Thyroiditis, Disease of the Month, September 1962.

SECTION IX

TESTS FOR RHEUMATOID ARTHRITIS

A. INTRODUCTION

Rheumatoid Arthritis is a disorder of unknown etiology, without obvious precipitating cause, and with an unpredictable course.

1. The test for the rheumatoid factor in use in 1932 was Group A hemolytic streptococcus agglutination. Subsequently, the sensitized sheep cell agglutination was used. Sensitivity and specificity of these procedures was not uniform, but arbitrary levels have been assigned as positive or negative in each and have some basis in comparative tests. Doubtful results in the streptococcus agglutination were considered negative. In the differential sheep cell agglutination, a differential titer of 16 and above, or a sensitized titer of 256 or above were considered to be positive.

2. Latex Fixation Test:

Antigen antibody reaction may also be made visible if carried out in the presence of latex or colloidan particles. Polyvinyl toluene latex of 0.81 microns from the Dow Chemical Co., supplied as an 11% suspension, is diluted about 1:10, filtered through paper and adjusted to such density that a 1:100 dilution in saline borate buffer gives a pH of 8.2.

3. In tests for the rheumatoid factor serial dilutions of serum in borate or glycine buffer are prepared using 1 ml. per tube. To each dilution is added 1 ml. of a 1:100 latex suspension containing 0.25 mg. gamma globulin fraction II, in borate or glycine buffer. Incubation is at 56°C. for two hours, centrifuge at 3300 rpm for three minutes and the test result is the degree of agglutination. Controls of saline instead of serum are used. The latex fixation test is positive in 71% of patients suffering from rheumatoid arthritis as compared to 2% of controls and paralleled other tests. This method has been used with Type VII anti-pneumococcal rabbit serum and SVII, and with Anti-C reactive protein and serum from patients with rheumatic fever and other related conditions.

4. The test has also been used with calf thymus nucleoprotein and sera of patients with lupus erythematosus. In applying the test to other systems the concentration of latex is adjusted to give optimal results. Grey, Tupper, and Rawse attribute the reaction of the rheumatoid sera in the latex fixation test to a heat labile glycoprotein. However, the studies of others have shown that heating gamma globulin solutions at 62°-65°C. causes agglutination and increases the capacity to precipitate with sera of rheumatoid arthritis patients. It is probably this reaction which the latex fixation test makes visible. Prozones in the latex

fixation test are dependent on calcium and a temperature labile substance resembling components of complement. Inactivation of sera from patients with rheumatoid arthritis is recommended.

B. RA SLIDE TEST

The procedure presently employed in the Naval Medical School's Serology Laboratory to detect the presence of the rheumatoid arthritis factor is the rapid slide test.

1. Materials and Preparations

The RA test is available as a kit containing latex globulin reagent, positive control serum, negative control serum, glycine-saline buffer diluent, and a divided glass slide.

a. Latex-Globulin Reagent is prepared from polystyrene latex and human gamma globulin. The reagent is processed to give rapid and clear-cut reactions with serums from patients with rheumatoid arthritis and is employed in any laboratory as a simple screening test for the rheumatoid factor. This type of screening test is 96% effective.

b. Control Serums:

(1) Positive control serum contains rheumatoid serum in a dilution of 1:20 and is used without further dilution.

(2) Negative control serum contains normal human serum in a dilution of 1:20 and is used without further dilution.

(3) Both control serums contain sodium azide 0.1% as a preservative but should be kept at 2^{o} to 10^{o}C. when not in use.

c. Glycine-Saline Buffer Diluent is a sterile solution of glycine and sodium chloride at pH 8.2. This solution contains no preservative and should be stored at 2^{o} to 10^{o}C. after the bottle has been entered. Do not use solution if it becomes cloudy.

d. The divided slide must be kept clean for satisfactory results.

2. Procedure

a. Prepare an approximately 1:20 dilution of the serum under test by adding 1 drop of serum to 1 cc. of Glycine-Saline Buffer Diluent.

b. Place 1 drop of diluted serum specimen in a rectangle of the divided slide.

c. Add 1 drop of Latex-Globulin Reagent, mix with an applicator or toothpick, and spread over an area of approximately 20 x 25 mm.

d. Prepare positive and negative controls, each with 1 drop of appropriate RA-TEST Control Serum (use without further dilution) and 1 drop of Latex-Globulin Reagent. Use separate applicator or toothpick for each mixture.

e. Tilt the slide from side to side for 1 minute and observe for macroscopic clumping.

3. Reading RA Slide Test Results

Recommended method of reading the test is as follows:

a. Negative-Smooth suspension with no visible flocculation, as shown by negative control.

b. Weakly Reactive - Visible flocculation but with small aggregates or partial clumping.

c. Reactive - Visible flocculation with large aggregates and complete clumping (clean background), as shown by positive control.

d. Serums containing rheumatoid factor produce visible flocculation of RA-TEST Reagent whereas a smooth suspension will be observed in a negative reaction. In positive tests, visible flocculation usually occurs in a few seconds.

e. If the screening test for the rheumatoid factor is positive or weakly positive we then set up the Rose Test.

C. ROSE TEST

1. Principle:

A differential agglutination titer of normal and sensitized sheep cells can be demonstrated with serum from patients having rheumatoid arthritis. A higher proportion of positive tests occurs as the disease advances or increases in severity. The test remains positive on remissions due to hormone treatment. This test is negative for arthritis with psoriasis and juvenile arthritis.

2. Material and Preparations

a. Patient's serum inactivated at 56°C. for 30 minutes.

b. 1% Normal Sheep Cells - These can be prepared by diluting the 2% suspension as used in the Davidsohn test with an equal volume of physiological saline. Increase the amount accordingly to the number of tests run. If one test is run the amount needed with control is 5.5 ml. (See Section X, paragraph 3-c, page 120.)

c. 1% Sensitized Sheep Cells - In order to prepare a 1% sensitized sheep cell suspension it is necessary to dilute an equal volume of 2% sheep cells with an equal volume of hemolysin dilution as prepared from the agglutination titration.

Example: 2+ agglutination in tube 10 of the Rose Test Agglutination titration is 1:1600 (double the agglutining unit to 1:3200). From stock 1:100 hemolysin prepare a dilution of 1:3200 by adding 0.1 of the 1:100 stock hemolysin to 3.1 ml.of saline. Add an equal part of 2% sheep cells to this dilution.

(1) In a wire rack place 14 test tubes (13 x 100 ml.) numbered 1 to 14 . Prepare agglutination titrations from 1:100 to 1:4000 of hemolysin to be contained in a volume of 0.5 ml., as follows:

Table 32.

Tube No.	Hemolysin (1:100) ml.	0.9% NaCl ml.	Process	Dilution Titer
1	0.5	None	None	1:100
2	0.5	0.5	Mix, discard 0.5	1:200
3	0.5	1.0	Mix, transfer 0.5 to tube 6, discard 0.5	1:300
4	0.5	1.5	Mix, transfer 0.5 to tube 7, discard 1.0	1:400
5	0.5	2.0	Mix, transfer 0.5 to tube 8, discard 1.5	1:500
6		0.5	Mix, transfer 0.5 to tube 9	1:600
7		0.5	Mix, transfer 0.5 to tube 10	1:800
8		0.5	Mix, transfer 0.5 to tube 11	1:1000
9		0.5	Mix, transfer 0.5 to tube 12	1:1200
10		0.5	Mix, transfer 0.5 to tube 13	1:1600
11		0.5	Mix, transfer 0.5 to tube 14	1:2000
12		0.5	Mix, discard 0.5	1:2400
13		0.5	Mix, discard 0.5	1:3200
14		0.5	Mix, discard 0.5	1:4000

(2) Add 0.5 ml. of 1% Sheep Cell suspension of washed sheep cells in each of test tubes 1-14.

(3) Place titration in a 37°C water bath for 1 hour.

(4) Remove and place rack in cold water bath in a refrigerator at 6°-10°C overnight.

(5) Remove from refrigerator and read immediately agglutination of 2+ or greater to establish agglutinating hemolysin titer for the Rose Sheep Cell agglutination Test.

(6) To establish the agglutining unit, double the dilution and mix with the 0.9% NaCl as follows:

Table 33.

Tube No.	Agglutination	Hemolytic dilution used	To prepare dilution 1:100 Hemolysin (ml.)	Saline (ml.)
1	1:100	1:200	1	1.0
2	1:200	1:400	1	3.0
3	1:300	1:600	1	5.0
4	1:400	1:800	1	7.0
5	1:500	1:1000	1	9.0
6	1:600	1:1200	1	11.0
7	1:800	1:1600	1	15.0
8	1:1000	1:2000	1	19.0
9	1:1200	1:2400	1	23.0
10	1:1600	1:3200	1	31.0
11	1:2000	1:4000	1	39.0
12	1:2400	1:4800	1	47.0
13	1:3200	1:6400	1	63.0
14	1:4000	1:8000	1	79.0

(7) In order to prepare a 1% sensitized sheep cell suspension it is necessary to dilute an equal volume of 2% sheep cells (washed) with an equal volume of hemolysin dilution as prepared from the agglutination titration.

3. Procedure for Rose Agglutination Test

a. Using 12 x 75 mm. test tubes, place two rows of 10 tubes each plus two control tubes (one for the normal sheep cell test and one for the sensitized sheep cell test).

b. Add 0.5 ml. of 0.9% saline to each tube of each row and the control tubes.

c. Add 0.5 ml. of serum to be tested to the first tube of each row.

d. After making serial dilutions of the patient's serum by mixing the patient's serum and saline in the first tube, then transferring 0.5 ml. to the next tube, continue mixing and transferring to the tenth tube, and discard 0.5 ml. from the tenth tube. This is done in each row.

e. Control tubes contain only saline.

f. To each tube in the front row and control tube add 0.5 ml. of normal sheep cells.

g. To each tube in the back row and control tube add 0.5 ml. of sensitized sheep cells.

h. Incubate the tubes in a 37°C. water bath for 1 hour.

i. Place in water bath in refrigerator at 6° to 10°C. and leave overnight.

j. Remove from refrigerator and read.

k. Final dilutions are 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048.

4. Reporting the Rose Test

The result of the test is reported as the titer of sensitized cells divided by the titer of the normal cells.

a. Report as sensitized titer 1:512 Normal titer 1:8 values 64 (512 divided by 8 = 64.)

b. Any answer up to and including 8 is considered negative.

5. Interpretation of the Rose Test

The result of the Rose Test, in order to indicate the probability of Rheumatoid Arthritis, must show a dilution titer of agglutination greater than 1:8. Out of 200 tests 96 showed agglutination values greater than 1:8. These two tests are considered to be highly effective when performed together to aid in the diagnosis of Rheumatoid Arthritis.

The Rose Test performed on sera containing the antibody has proved to be 100% effective.

Dates		Test	Total Tests	Positive Tests	Total Months	Average per month
From:	5 March 60	RA Slide	1636	200	30	55
To:	1 Oct 62	Rose Test	200	200	30	7

D. REFERENCES

Ball, J., Block, K.J., Burch, T.A., Kellgren, J.H., Lawrence, J.S., Tsigalidon, V.: Comparative studies of serologic test for rheumatoid disease. II. A comparison of the benlonite flocculation test and the sensitized sheep cell agglutination test. Arthritis Rheum. 5:61-9, Feb. 1962.

Ball, J., DeGraaf, R., Valkenburg, H.A., Boerma, F.W.: Comparative studies of a latex test and two erythrocyte agglutination tests in a random population sample. Arthritis Rheum. 5:55-60, Feb. 1962.

Christian, C.L: Serological reactions: The rhematoid factor in Rheumatoid Arthritis. Bull.N.Y.Acad.Med. 38:579-86, Sept. 1962.

Hall, A.P.: Serologic tests in rhematoid arthritis. Med.Clin.N.Amer. 45:1181-96, Sept. 1961.

Serologic reactions in rhematoid arthritis. Brit.Med.J. 5232:1097, 15 April 1961.

SECTION X

HETROPHILE ANTIBODY TEST FOR INFECTIOUS MONONUCLEOSIS

A. INTRODUCTION

Infectious mononucleosis is usually a benign disease, probably of viral origin. Its importance is seen in the lists of differential diagnostic possibilities to which it is related and to the difficulty in making a clinical diagnosis. The diagnosis is made by clinical suspicion, the presence of atypical lymphocytes in the peripheral smear and the presence of elevated heterophile antibody titers.

The serologic diagnosis of infectious mononucleosis is based on the heterophile antibody. Heterophile antibodies are a non-specific group of antibodies capable of reacting with numerous different plant or animal tissues. An example is the reaction between the antibody present in normal human beings and sheep red cells (present even when no prior immunization or exposure to the antigen is known). In infectious mononucleosis high titers of heterophile antibodies are present in the serum. The antibodies are separated into Forssman and non-Forssman types. The Forssman antibody is removed from serum by absorption with a guinea pig kidney preparation. The non-Forssman antibody is removed from serum by absorption with a beef red cell preparation. The hetrophile antibody related specifically to infectious mononucleosis is the non-Forssman type.

B. PRESUMPTIVE DAVIDSOHN TEST

1. Principle

The test is based on the agglutination of 2% sheep erythrocytes by all heterophilic antibodies in the serum of patients with infectious mononucleosis, or without infectious mononucleosis.

2. Indication

The presumptive test is indicated in cases where the clinical or the hematologic findings or both suggest infectious mononucleosis.

3. Materials

a. Test tubes, 75 mm. x 10 mm.

b. Serum inactivated for 30 minutes at 56oC., to inhibit complement.

c. A 2% suspension in saline of sheep red cells washed three times in 0.9% CPRG sodium chloride. The sheep red cells should be not less than 48 hours old and not older than 1 week. They should be used only if the third washing gives a perfectly colorless supernate. The cells should be washed and the suspension prepared and used on the same day.

d. One Kahn rack.

e. Pipets, 1 ml. and 5 ml.

4. Procedure

a. Set up a row of 11 tubes.

b. Add 0.4 ml. of 0.9% CPRG sodium chloride to the first tube and 0.25 ml. to all other tubes.

c. Add 0.1 ml. of serum to the first tube, mix and transfer 0.25 ml. to the second tube, and so on, until the tenth tube is reached. Mix and discard 0.25 ml. from the tenth tube. Serum dilutions are 1:5, 1:10, 1:20, and so forth.

NOTE: The last (11th) tube is a control tube containing only saline and sheep cells.

d. Add 0.1 ml. of 2% suspension of sheep red cells to all tubes. Shake the tubes. Final dilutions are 1:7, 1:14, and so on.

e. Shake rack vigorously for 10 seconds.

f. Let stand at room temperature for 2 hours.

5. Reading Results

a. Results are read after shaking the test tubes to resuspend the sediment. Check with the naked eye, using a single light source and the concave side of a microscope mirror. When there is no visible agglutination as compared to the control tube, the end-point is reached.

b. When speed is indicated, the reading may be done after 15 minutes. If the result is positive (agglutination in dilutions 1:224 or higher), the test may be considered completed except that the final titer will be higher after 2 hours' incubation. If negative (titer less than 1:224), repeat the reading at intervals as frequent as convenient. Final negative result (titer less than 1:56) should not be recorded until after 2 hours' incubation. If speed is not a factor, it may be more convenient to read results at the end of 2 hours.

6. Interpretation

In the presence of clinical and/or hematologic findings suggestive of infectious mononucleosis, titers of 1:224 or higher in the presumptive test support the diagnosis of infectious mononucleosis. If the titer of the presumptive test is less than 1:224 in the presence of clinical and/or hematologic findings suggestive of infectious mononucleosis; or, if the titer of the presumptive test is 1:224 or higher in the absence of clinical and hematologic findings suggestive of infectious mononucleosis; or, if the patient has a history of a recent horse serum injection, the result of the presumptive test should be checked by the differential test (see below).

C. DIFFERENTIAL DAVIDSOHN TEST

1. Principle

The heterophilic antibodies (antisheep agglutinins) in infectious mononucleosis are not of the Forssman type. They are not absorbed by suspensions of guinea pig kidney but are readily and completely absorbed by beef red cells. The heterophilic antibodies in normal persons, in horse serum sensitization, and in a variety of other conditions are of the Forssman type and are readily and completely absorbed by suspensions of guinea pig kidney. In horse serum sensitization absorption with beef red cells removes the sheep agglutinins readily and completely, whereas in normal persons and in patients with diseases other than infectious mononucleosis or horse serum sensitization, sheep agglutinins are frequently removed only partially by the beef cell antigen.

2. Materials

a. Test tubes, 75 mm. x 10 mm.

b. Graduated centrifuge tubes

c. Serum, inactivated for 30 minutes at 56°C. to inhibit complement.

d. A 2% suspension in saline of sheep red cells washed three times in 0.9% CPRG sodium chloride. The sheep red cells should be not less than 48 hours old and not older than 1 week. They should be used only if the third washing gives a perfectly colorless supernate. The cells should be washed and the suspension prepared and used on the same day.

e. Antigen - either Guinea Pig Kidney antigen or Beef Erythrocyte Antigen, as applicable to test being performed.

3. Differential Test Absorption with Guinea Pig Kidney Antigen

a. Indications include:

(1) A titer of antisheep agglutinins (1:112 or less), as determined by the presumptive test, in cases suspected of having infectious mononucleosis.

(2) A titer of antisheep agglutinins of 1:56 or higher in patient without clinical or hematologic findings of infectious mononucleosis.

(3) A history of a recent injection of a horse serum in a patient with a titer of antisheep agglutinins of 1:56 or higher as determined by the presumptive test.

b. Preparation of Guinea Pig Kidney Antigen:

This antigen is manufactured by sacrificing 100 guinea pigs. From the lifeless animals the kidneys are removed and placed into 50 ml. of 0.9% saline. Continue washing the kidneys in fresh 0.9% saline solution until the washings are free of blood erythrocytes. The G.P. kidneys are then placed into an osterizer and ground to a fine pulp. The pulp is weighed exactly and once the exact weight has been determined the correct mathematical calculation is performed to make a 20% suspension in 0.9% saline solution. The total volume is marked on the outside of the flask, for there will be a loss by evaporation after boiling. The 20% kidney suspension is boiled for 1 hour in a water bath at 100°C. After boiling add sufficient quantity of distilled water to bring contents to the marking on the flask to adjust volume to a 20% suspension. Allow the suspension to set at room temperature for 1 hour. To each 100 ml. of 20% G.P. Antigen you would add 0.5 ml. of pure phenol as a preservative. This suspension is now completed and ready to use. When stored at 6° to 10°C., it will last until the supply is exhausted.

c. Procedure for absorption with Guinea Pig Antigen:

(1) Pour into a graduated centrifuge tube 1 ml. of the thoroughly shaken suspension of guinea pig antigen. (SHOULD NOT BE PIPETTED.)

(2) Add 0.2 ml. of inactivated serum.

(3) Shake well and let stand for 3 minutes, at room temperature.

(4) Centrifuge at 1,500 rpm for 10 minutes.

(5) Remove the supernatant fluid carefully with a capillary pipet. Make sure to transfer only the clear supernate without particles.

(6) Set up a row of 10 tubes (75 mm. by 10 mm.). Add 0.25 ml. of 0.9% CPRG solution of sodium chloride to all tubes except the first.

(7) Add 0.25 ml. of the supernatant fluid described above (see (5)) to the first tube.

(8) Add 0.25 ml. of the supernatant fluid to the second tube. Mix and transfer 0.25 ml. to the third tube, and so on. Discard 0.25 ml. from the last tube. The serum dilutions are 1:5, 1:10, 1:20, and so forth.

(9) Add 0.1 ml. of a 2% suspension of sheep cells. Final dilutions are: 1:7, 1:14, 1:28, and so on. Shake vigorously for 10 seconds.

(10) Let stand at room temperature for 2 hours.

d. Reading and Reporting Results:

Results are read after shaking the test tubes to resuspend the sediment; proceed as in the presumptive test (see B-5 above).

(1) When speed is indicated, the reading may be done after 15 minutes. If the test is reactive, which means agglutination in the same dilution as the presumptive test or in not more than three dilutions or tubes below that of the titer of the presumptive test, then the test can be reported as positive for infectious mononucleosis. If the result is negative, which means more than three tubes difference in the agglutination as compared with the titer of the presumptive test, repeat the reading at intervals as frequent as convenient. Final negative results should not be recorded until after 2 hours' incubation.

e. Interpretation of Differential Test with Guinea Pig Kidney Antigen

The differential test for infectious mononucleosis is positive if the titer of antisheep agglutinins after absorption with guinea pig kidney is not more than three tubes lower than the titer of the presumptive test.

Examples:

Table 34. Reporting Results.

Titer		
Presumptive Test	Differential test after absorption with guinea pig kidney	Results
1:224	1:112	Reactive for infectious mononucleosis
"	1:56	" " " "
"	1:28	" " " "
"	1:14	Non-reactive for infectious mononucleosis
"	1:7	" " " "
1:56	1:56	Reactive for infectious mononucleosis
"	1:28	" " " "
"	1:14	" " " "
"	1:7	" " " "
1:28	1:28	Non-reactive for infectious mononucleosis
"	1:14	" " " " "

In all reactive tests the absorption with beef cells, if done, will show complete or almost complete removal of agglutinins. In the non-reactive tests absorption with beef cells, if done, may show incomplete removal of the agglutinins.

4. Differential Test Absorption with Beef Erythrocyte Antigen

a. Indications:

It may be preferable to do the absorption with guinea pig kidney and with beef cell antigens in every case. The absorption with beef cells is essential in cases of patients with elevated titers of antisheep agglutinins if no clinical or hematologic findings are present suggestive of infectious mononucleosis. In most of these instances the antisheep agglutinins will be completely removed by guinea pig kidney antigen, but, in rare instances, the absorption may be incomplete. The use of the beef cell antigen will then be decisive.

b. Preparation of Beef Erythrocyte Antigen:

Beef erythrocytes are washed three times using 0.9% chemically pure reagent grade sodium chloride. After the third washing the supernatant fluid must be colorless. If a slight reddish color is present in the supernatant after the third wash this is an indication the cells are too fragile and should not be used. If the supernatant is clear on the third wash you remove the supernatant without disturbing the packed cells. Take one volume of these washed, packed erythrocytes and suspend them into four volumes of saline and then this suspension is boiled for 1 hour in a water bath at 100°C. Loss by evaporation is made up with distilled water. Phenol is added as a preservative. You must add .5 ml. of pure phenol to each 100 ml. of beef erythrocyte antigen. The antigen is stable until the supply is exhausted when stored at 6° to 10°C.

c. Procedure for Absorption with Beef Erythrocytes Antigen:

(1) Pipet into a graduated centrifuge tube 1 ml. of the thoroughly shaken suspension of beef erythrocytes antigen.

(2) Add 0.2 ml. of inactivated serum.

(3) Shake well for 10 seconds and let stand for 3 minutes at room temperature.

(4) Centrifuge at 1,500 rpm for 10 minutes.

(5) Remove the supernatant fluid carefully with a capillary pipet. Make sure to transfer only clear supernate without particles.

(6) Set up a row of 11 tubes (75 mm. by 10 mm.). Add 0.25 ml. of 0.9% sodium chloride to all tubes except the first.

(7) Add 0.25 ml. of the supernatant fluid (as described in (5) above) to the first tube.

(8) Add 0.25 ml. of the supernatant fluid to the second tube. Mix and transfer 0.25 ml. to the third tube, and so on. Discard 0.25 ml. from the tenth tube. The serum and dilutions are 1:5, 1:10, 1:20, and so forth.

NOTE: The 11th tube is the control tube and contains 0.25 ml. of saline and 0.1 ml. of sheep red cells.

(9) Add 0.1 ml. of a 2% suspension of sheep red cells to all tubes. Final dilutions are 1:7, 1:14, 1:28, and so forth. Shake vigorously for 10 seconds.

(10) Let stand at room temperature for 2 hours.

d. Reading and Reporting Results:

Results are read after shaking the test tubes to resuspend the sediment; proceed as indicated in the presumptive Davidsohn. (See B-5.)

(1) When speed is indicated, the reading may be done after 15 minutes. If agglutination is present in the same dilution as in the presumptive test or in dilutions from two to three tubes lower, the result is negative for infectious mononucleosis. If no agglutination is present, repeat the reading at intervals as frequent as convenient. If no agglutination is present, final results should not be recorded until after 2 hours' incubation.

e. Interpretation of Differential Test with Beef Antigen

The test for infectious mononucleosis is reactive in a serum in which the absorption with guinea pig kidney failed to remove the antisheep agglutinins completely and in which the beef cell antigen completely removed the antisheep agglutinins.

Examples:

Titer			Result
Presumptive Test	Differential test after absorption with:		
	guinea pig kidney	beef erythrocytes	
(a) 1:224	0	1:112	Nonreactive for infectious mononucleosis
(b) 1:448	1:224	1:224	Nonreactive for infectious mononucleosis
(c) 1:448	1:224	0	Reactive for infectious mononucleosis

SUMMARY

The presumptive test for infectious mononucleosis is a quantitative nonspecific test. Titers of 1:224 or higher make possible a presumptive diagnosis of infectious mononucleosis in the presence of clinical and/or hematologic findings suggestive of the disease.

The differential test consists of determination of the antisheep agglutinins after (1) absorption with guinea pig antigen and (2) absorption with beef cell antigen.

The differential test is specific for infectious mononucleosis. A positive test is indicated by (1) incomplete removal of antisheep agglutinins by guinea pig kidney and (2) complete removal of the antisheep agglutinins by beef cells.

D. REFERENCES

Bender, C.E.: Interpretations of hematologic and serologic findings in diagnosis of infectious mononucleosis. Ann. Int. Med. 49:852, 1958.

Davidsohn I.: Serologic diagnosis of infectious mononucleosis. JAMA 108:289, 1937.

Davidsohn, I., Lee, C.T.: The laboratory in the diagnosis of infectious mononucleosis. Med. Clin. Na.:46:125, 1962.

Davidsohn, I., Stern, K., Kashewagi, C.: The differential tests for infectious mononucleosis. Am. J. Clin. Path. 21:1101, 1951.

Davidsohn, I. and Walker, P.H.: Nature of heterophilic antibodies in infectious mononucleosis. Am. J. Clin. Path. 21:1101. 1951.

Paul, J.R. and Bunnel, W.W.: Presence of heterophilic antibodies in infectious mononucleosis. Am. J. M. Sci. 183:90, 1932.

SECTION XI

TEST FOR COLD AGGLUTININS

A. INTRODUCTION

The serum of certain individuals may agglutinate homologous-type cells when the two are mixed and kept at refrigerator temperatures or sometimes even at room temperature. This phenomenon is due to so-called cold agglutinins. These cold agglutinins are nonspecific and clump cells regardless of the blood group. When cells of the same person are clumped by his own serum, they are called auto-agglutinins. The cell clumps break up when the mixtures are brought to body temperature. Cold agglutinins are found in a considerable number of cases of primary atypical pneumonia and less frequently in other acute infections and certain pathologic conditions.

B. MATERIALS

1. Standard test tubes (75 mm. x 10 mm. - inside diameter)

2. Saline solution, 0.9%

3. Sodium citrate solution, 2.5%

4. Patient's cells and serum.

C. COLLECTION OF BLOOD SPECIMEN

As most cold agglutinins are also auto-agglutinins, extreme care must be used in preparing the serum for the test.

1, Never use a cold syringe to draw the blood specimen. The syringe may be warmed by enclosing it in the palm of the hand for a few minutes.

2. The tube to receive the blood should be warm.

3. After the blood specimen is obtained, it must be kept warm while in the process of delivering it to the laboratory. This can be accomplished by transporting the tube of blood in a glass of warm water, or the technician may carry the tube of blood in his shirt pocket.

4. The laboratory technician should place the tube immediately in a 37°C. water bath or incubator until it is centrifuged.

5. After the serum is separated from the cells, it should be stored in a refrigerator until the test is set up. If the specimen is chilled before the serum is

separated the cells may absorb the cold agglutinins from the serum, and in centrifuging, the cold agglutinins will be partly or completely removed with the packed cells or clot.

D. PROCEDURE

1. Collect approximately 7 ml. of blood by venipuncture.

2. One drop of blood (from the needle with the bevel down) is added to a tube containing 10 ml. of a 2.5% sodium citrate solution. Mix by inverting the tube.

3. Place about 5 ml. of blood in a clean, dry test tube and allow to clot. When the clot has formed, centrifuge and separate the serum.

NOTE: The serum must be fresh because old serum may give falsely negative results.

4. Centrifuge the patient's cells for 15 minutes at 2,000 rpm in a graduated centrifuge tube, pour off the supernatant fluid, and then make a 2% suspension in 0.85% saline solution.

NOTE: Type O cells from a healthy individual may be prepared as above and used in place of the patient's cells, when only serum is received.

5. Place 11 small test tubes (Kahn) in a rack.

6. Add 1.5 ml. of 0.9% saline to the first tube and 1 ml. to each of the remaining 10 tubes.

7. Add 0.5 ml. of the patient's serum to the first tube. Mix thoroughly and transfer 1 ml. to the second tube, mix and transfer 1 ml. to the third tube, and so on to the tenth tube from which 1 ml. is discarded. The eleventh tube is a control and contains no serum.

8. Add to each tube 0.1 ml. of the previously prepared cell suspension (see #4). After shaking each tube to insure thorough mixing the rack is placed in an ice bath, which is in turn placed in a refrigerator overnight.

E. READING OF RESULTS

Agglutination, if present, will disappear in one hour at room temperature, so the reading must be made immediately.

1. Remove one tube at a time from the ice bath and examine for agglutination. Report the titer as the highest dilution showing agglutination of one plus or more. The serum dilution in the first tube is 1:4, in the second tube 1:8, and so on.

2. If there is no agglutination in any of the tubes, the test is reported as negative.

3. If positive, allow the tubes to stand at room temperature for an hour and then make a second reading. If the reaction is a true cold agglutination, the clumps will disperse.

4. Purpose of control is to check the cells and reactions to prevent non-specific agglutination.

F. INTERPRETATION

One test is not sufficient; serial tests should be run to observe titer changes. A titer of 1:32 or higher is suggestive of atypical or virus pneumonia. NOTE: This disease, when caused by the Eaton agent, is accompanied, in approximately 40% of the cases, by elevated titers of cold agglutinins. A titer of 1:1,024 or above is not uncommon in that disease. Elevated titers of agglutinins may also occur in other conditions, such as acquired hemolytic jaundice, cirrhosis and other diseases of the liver, chronic sepsis, leishmaniasis, and blackwater fever.

G. REFERENCES

Chanock et al: Eaton Agent Pneumonia, JAMA 175:213, 1961.

Feller, A.E. and Hilteman, M.R.: Primary Atypical Pneumonia in American Public Health Associates. Diagnostic Procedures for Virus and Rickettsial Diseases, 1956, pp. 263-280.

SECTION XII

DEFINITION OF SEROLOGY TERMS

Agglutinins - An antibody-like substance found in the serum and other body fluids which will cause agglutination of cells containing the opposing agglutinogen.

Agglutinogens - An antigenic substance found in red blood cells (bacteria) which causes agglutination of the cells. Agglutinogens, when injected into another person incite the formation of opposing agglutinins.

Antigen - Any substance which when introduced into the body stimulate the formation of antibodies.

Antibody - The substance produced in the animal body against an antigen. An antibody exerts a specific antagonistic influence on the substance that incited or stimulated its formation.

Autohemagglutinin - An agglutinin which will agglutinate an individual's own erythrocytes.

Cardiolipin-Antigen - An antigen containing cardiolipin, lecithin, and cholesterol. This is disolved in alcohol and is a refined antigen.

Cholesterol - Cholesterol is a lipoid-like substance used to increase the sensitivity of antigen. Cholesterol acts mainly as an inert core around which the antigenic lipids are spread.

Chyle - Emulsified fat appearing in the blood after a meal. Chyle in the serum gives it a white, cloudy appearance.

Colloid - The suspension throughout a liquid of particles smaller than 5 microns in diameter.

Complement - A thermolabile, ferment-like substance which in combination with hemolysin causes lysis of the erythrocytes.

Differential - Differentiate. Tell the difference between. Obtain exact, precise information.

Dispersed - Scattered.

Emulsion - The suspension of fine globules of one liquid in another.

Flocculation - The formation of visible particles or flakes in a liquid.

Globulin - That fraction of the protein part of the blood or serum which contains the antibodies.

Hemolysis - Is the word given to the reaction which takes place in vitro when hemolysin and complement react together to destroy erythrocytes.

Hemolysin - An antibody present in serum which will cause lysis of its opposing antigen in the presence of complement. Example: Anti-sheep cell antibody produced in a rabbit by injecting rabbits with sheep erythrocytes will cause sheep cells to lyse extruding hemoglobin if complement is present.

Heterophile Antigens - Are substances which stimulate production of antibodies capable of reacting with tissues from other animals, fish, or even plants.

Immunization - Is the term loosely applied by laboratory workers to the process of antibody production for experimental or therapeutic use. Immunization antigens need not be pathogenic; in fact most theoretic investigations of the antigen-antibody reaction employ nonpathogenic agents such as erythrocytes, egg albumin, serum proteins.

Immunity - The ability of living individuals to resist or overcome infections.

Interpretation - Explanation or translation of results in accordance with established rules for reporting

Iso-Agglutinin - An agglutinin which causes agglutination of cells of the other members of the same species.

Lecithin - A complex nitrogenous substance found in brain, nerve, tissue, heart, and egg yolk.

Lipids - They are considered to be esters of organic acids with various alcohols, plus other radicals such as phosphate and sulfates, nitrogenous bases, etc.

Lipoidal antigen - A crude antigen prepared from alcoholic extract of dehydrated beef heart that has been processed first with ether. Lipoids are fat-like substances.

Micro-Flocculation - The formation of microscopically visible particles or flakes in a liquid.

Optimum - Best or most suitable.

pH The property of hydrogen or the value of the hydrogen ion in a substance establishing the acidity or alkalinity of the substance.

Physiological salt solution - Saline, normal saline, NaCl. This solution is a .9% solution of chemically pure, reagent grade sodium chloride crystals. It resembles most of the animal fluids in action, density and osmotic pressure.

Qualitative - Concerning quality. Measuring presence or absence.
Quantitative - Concerning quantity. Measuring how much.

Reagin - Reagin is the name given to the antibody-like substance found in syphilitic serum. It is created in response to:

A. Treponema pallidum
B. The body tissues which have been altered by its invasion.

Selectivity - The ability of a test to respond with a desired reaction so as to exclude as many of the non-reactive cases as possible.
Sensitivity - The ability of a test to detect as nearly 100% of the reactive cases as possible.

Serodiagnosis - Diagnosis made through serologic procedures.
Serology - Serology is a laboratory means of measuring the potential protective antibodies in the individual. Serological technics also provide diagnostic methods for the identification of infectious agents as well as identifying antigens or antibodies not related to disease.
Serum - The clear liquid which separates from the clot when blood has coagulated. For serological procedures, it is imperative that it contain no red blood cells.
Solution - A complete, homogeneous, and molecular incorporation of one substance in another.
Specificity - The ability of a test to determine a reaction of a particular character.
Specimen - A sample or small quantity of a substance which shows the kind of quality of the whole, such as a blood or urine specimen.
Supplementary - In addition to.
Suspension - The state of a solid when its particles are mixed with, but undissolved in, a fluid.

Titer - The highest (greatest) dilution of serum which still has sufficient antibody present to react with a corresponding antigen. For example, 1:8 or 1 in 8 strength of antibody.

Treponema pallidum - The cork-screw-shaped micro-organism which causes the disease syphilis and incites the formation of the reagin antibodies.